

1998

Physiological consequences of astrocyte-to-neuron signaling

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Physiological consequences of astrocyte-to-neuron signaling

by

Rita Prabhakar Sanzgiri

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Genetics

Major Professors: Philip G. Haydon and Drena L. Dobbs

Iowa State University

Ames, Iowa

1998

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ACKNOWLEDGMENTS

I take this opportunity to thank the Iowa State University, the Interdepartmental Genetics program and the Department of Zoology and Genetics for allowing me to pursue my Ph.D. I must thank Phil Haydon, my major professor for his guidance throughout my graduate career, and for providing me with an excellent work environment in his laboratory. I must also thank Drena Dobbs, my co-major professor, and my committee members Susan Carpenter, Kristen Johansen, Linda Ambrosio and Sheldon Shen for their constant encouragement and advice.

I thank all the members of the Haydon lab for making my graduate life truly enjoyable. I remember my first day at the laboratory as an unsuspecting molecular biologist who walked into some seasoned electrophysiologists like Fred Bahls and Trent Basarsky! I have immensely enjoyed all those battles in which I had to valiantly, and sometimes unsuccessfully, defend the molecular biological approach with the backing of Yu Fang and Sally Durgerian. I must thank Louis Trudeau, with whom I enjoyed doing experiments. Lynn Smith and Gerry McDade for providing me with cells and laughs whenever I needed them. Bob Doyle for his reassuring presence and Vlad Parpura for just being himself! I thank Barbara Innocenti and Mary Rose for looking after me in my insane thesis-writing phase. It was the calcium-rich orange juice that kept me going, Barbara! Gracias muchas to Alfonso Araque for his time and patience(!) in teaching me electrophysiology, and above all, for teaching me how to enjoy science and, yes I confess, for introducing me to "the Spanish bubble"! I acknowledge Sarah Rigdon, Linda Wild and Eileen Muff for their efficient administrative help. My special thanks to all my colleagues and friends from outside of the laboratory for making my years at ISU some of the best years of my life.

Finally, I would not have reached this stage without the support and encouragement of my family, in particular my aunt Yamuna Mokashi, my parents Suman and Prabhakar Sanzgiri, and my personal stabilizer, who kept me going through all those difficult moments, Craig Zeutzius.

ABSTRACT

The traditional view of the central nervous system (CNS) in which neurons are the only cells capable of active intercellular signaling while glial cells merely provide structural and trophic support to neurons, has been challenged by a number of new findings. Recent evidence shows that astrocytes, a subtype of glial cells, participate in inter-astrocyte signaling by using variations in their cytosolic Ca^{2+} levels (Cornell-Bell et al., 1990; Charles et al., 1991; Finkbeiner et al., 1992; Duffy and MacVicar 1995). Neuronal activity can trigger these Ca^{2+} increases in astrocytes indicating that neurons can signal to astrocytes (Dani et al., 1992; Porter and McCarthy, 1996; Pasti et al., 1997). Finally, the demonstration that Ca^{2+} elevations in astrocytes evoke glutamate release from them and subsequent glutamate-dependent Ca^{2+} elevations in adjacent neurons, have raised the exciting possibility that astrocytes may in turn, signal back to neurons (Nedergaard et al., 1994; Parpura et al., 1994; Charles et al., 1994; Hassinger et al., 1995; Pasti et al., 1997; Bezzi et al., 1998).

Although the existence of astrocyte to neuron signaling has been firmly established by these Ca^{2+} imaging studies, the physiological consequences of this signaling have not been elucidated. The work presented in this thesis, provides electrophysiological evidence for the astrocyte-neuron signaling and demonstrates that stimulation of astrocytes leads to Ca^{2+} increases in astrocytes which modulates neuronal currents and synaptic transmission in hippocampal cultured neurons. This effect is mediated by the neurotransmitter, glutamate which is released from the astrocytes. A variety of stimuli that increase the intracellular Ca^{2+} levels in astrocytes, such as mechanical or electrical stimulation and application of the neuroligand prostaglandin E_2 , generate a glutamate-dependent slow inward current (SIC) mediated through the NMDA and non-NMDA glutamate receptors and/or an increase in the frequency of the miniature synaptic currents through the NMDA receptors in adjacent neurons. Additionally, stimulation of astrocytes transiently reduces the amplitude of the

action potential-evoked postsynaptic current mediated through the metabotropic glutamate receptors.

This work proposes a new view of the nervous system in which neurons and astrocytes exist as a network in which bidirectional communication takes place.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Glia and neurons constitute the two major classes of cells in the central nervous system (CNS). The classical dogma has been that neurons are the sole participants in the intercellular signaling process required for higher cognitive functions like information encoding and processing, while glia play more passive roles of merely providing structural and nutritive support to neurons. In the last decade a number of new studies have significantly altered our understanding of the glial role in the CNS. It is becoming increasingly clear that glia, once described as "neuroglue" (Virchow, 1876) or "cement that binds the nervous elements together", may play more dynamic signaling roles. Recent data has introduced the idea that astrocytes, a subtype of glial cells, can participate in astrocyte-to-astrocyte, neuron-to-astrocyte and astrocyte-to-neuron communication. The goal of this study was to further investigate the recently discovered phenomenon of astrocyte-to-neuron signaling and determine its physiological consequences.

Inter-neuronal communication occurs through electrical signals

Neurons are considered the basic computational units of the nervous system (Ramon y Cajal, 1911). The key property of neurons that makes them suitable for this signaling role, is their electrical excitability (Adrian, 1946; Sherrington, 1947; Kuffler et al., 1984; Hille, 1992; Kandel et al., 1995). Individual neurons receive and transmit information in the form of potential changes produced by electrical currents across their membranes. These currents are carried by ions such as Na^+ , K^+ , Cl^- etc. The potential difference between the outside and inside of the neuronal cell membrane depends on the ionic concentration gradients across the membrane and on the relative permeabilities of these ions. Electrical signals which consist of changes in the membrane potential are generated by selective changes in the permeability of the membrane for specific ions.

Typically, the resting cell membrane potential of a neuron is about -60 mV, the inside being more negative with respect to the outside. Upon stimulation, the cell membrane may be depolarized (excitatory input), that is the membrane potential changes in the positive direction, the inside becoming less negative with respect to the outside. On the other hand, it may be hyperpolarized (inhibitory input), that is the membrane potential changes in the negative direction, the inside becoming more negative with respect to the outside. A neuron receives many such excitatory and inhibitory inputs from other neurons and is able to summate them. When the net magnitude of its depolarization reaches a critical level (threshold), the cell fires an action potential. Action potentials are regenerative changes in the membrane potential of a fixed amplitude, which are conducted over long distances without attenuation. The frequency of firing action potential conveys information about the strength of the stimulus and forms the universal language of nerve cells in all animals that have been studied so far.

The point of communication between two neurons at which the transfer of signal takes place is called the synapse and the process of this communication is called synaptic transmission (For reviews see, Trimble et al., 1991; O'Connor et al., 1994; Südhof, 1995). Two distinct modes of synaptic transmission are known, electrical and chemical. At electrical synapses, currents generated by an action potential in one neuron (presynaptic) spread directly to another (postsynaptic) by direct movement of ions through gap junctions between the two nerve terminals. At chemical synapses the presynaptic nerve terminal secretes a specific substance, the neurotransmitter, which diffuses across the synaptic cleft and binds to the transmitter receptors on the postsynaptic membrane, where it changes the permeability of the membrane to specific ions and causes ion fluxes. The secretion of the transmitter is thought to occur through exocytosis of transmitter-filled vesicles called synaptic vesicles which are present in the presynaptic terminal.

At a chemical synapse the sequence of events is as follows. Excitatory stimulation of the presynaptic neuron causes a depolarization of its cell membrane. If the depolarization reaches the threshold value the cell fires an action potential. The wave of action potential propagates along the axon, and reaches the nerve terminal where it depolarizes the

presynaptic cell membrane and opens the voltage-sensitive Ca^{2+} channels in the presynaptic cell membrane. The extracellular Ca^{2+} ions now enter through the open channels. The influx of Ca^{2+} ions triggers a cascade of synaptic proteins interactions which finally results in the fusion and exocytosis of the synaptic vesicles and release of the neurotransmitter into the synaptic cleft. The transmitter then diffuses through the synaptic cleft and binds to its receptors on the postsynaptic membrane where it alters the permeability of specific ions either directly by opening/ closing ion channels or indirectly through other signal transduction pathways. The flux of ions through the postsynaptic membrane gives rise to a postsynaptic current (PSC) which leads to a change in the postsynaptic membrane potential (PSP). Depending upon the ions involved the change may be excitatory, where the new membrane potential is brought closer to threshold (EPSP), or inhibitory where, the new membrane potential is brought away from the threshold (IPSP). For example, the neurotransmitter glutamate is excitatory since activation of the glutamate receptors gives rise to an EPSP by opening Na^+ or Ca^{2+} channels. Even at the resting state, in absence of the action potential, there is a basal level of transmitter release from the presynaptic neuron which can give rise to miniature EPSPs (mEPSPs) or miniature IPSPs (mIPSPs). These miniature PSPs (mPSPs) can be studied in the presence of tetrodotoxin, a deadly poison produced by the puffer fish, which suppresses action potentials by selectively blocking the Na^+ channels required for it. (The history of tetrodotoxin (Kao 1966), begins with the discovery of its effects by the Chinese emperor, Shan Nung (2838-2698 B.C.), who *personally tasted* 365 drugs while compiling a pharmacopoeia!)

The electrical activity of a neuron can be monitored by an electrode placed outside of a cell (extracellular recording) or by a microelectrode that actually penetrates through the cell membrane (intracellular recording). The electrode placement is made using a micromanipulator. All the recordings mentioned in the next chapters are of the intracellular type. Two electrodes are used to measure the potential difference across the membrane. The recording electrode, a fine glass capillary filled with the intracellular solution, is in contact with the cytoplasm and the reference electrode with the extracellular solution. The potential difference between them is amplified and fed to an oscilloscope. Cell currents are

measured while holding the membrane potential at a constant value or under conditions of voltage clamp. The constant voltage applied is called the command voltage and this is maintained by injecting a current into the cell to negate the net cellular current. When the command voltage is held at the resting membrane potential value, no current is recorded. However, cellular currents arise during excitation due to opening or closing of ion channels and the resulting ion fluxes. In order to keep the membrane potential at the previous resting value, the injecting current is now changed by an amount equal and opposite to the cellular current. Thus, under voltage clamp conditions, the cellular current can be measured in terms of the injecting current.

Glial cells perform a variety of functions in the CNS

Neurons in the CNS are intimately associated with glial cells which are further divided into various subtypes, astrocytes, oligodendrocytes and microglia, based on anatomical criteria. Historically, they have been viewed as a type of CNS connective tissue whose main function was to support the "true" functional cells of the brain, neurons. Significant advances in the understanding of the glial cell physiology, however, have made it clear that these cells perform a variety of important functions in the CNS. These include regulatory functions to control local concentrations of ions (Orkand et al., 1966; Quandt and MacVicar, 1986; Walz, 1989; Largo et al., 1996) and neuroactive substances (Mennerick and Zorumski, 1994; Largo et al., 1996; Rothstein et al., 1996; Bergles and Jahr, 1997), providing trophic support to neurons (Ramón y Cajal, 1911; Banker, 1980; Tsacopoulos and Magistretti, 1996), neuronal survival (Barde, 1989; Raff et al., 1993; Pfrieger and Barres, 1995) and differentiation (Engele et al., 1991; Gaul and Lübbert, 1992; Takeshima et al., 1994), neuronal guidance (Rakic, 1990; Bastiani and Goodman, 1986; Kuwada, 1986), neurite outgrowth (e.g., Noble et al., 1984; Johnson et al., 1989; Smith et al., 1990; LeRoux and Reh, 1994), and synaptic efficacy (Pfrieger and Barres, 1997).

Homeostasis: Neurons in the CNS are maintained in a highly regulated environment. Neuronal activity results in the release of neurotransmitters, extracellular pH shifts and changes in ionic concentrations. Glial cells play a major role in the homeostatic

mechanisms that restore the neuronal environment. For example, during neuronal activity, K^+ concentration in the extracellular space increases. Variations in extracellular K^+ can modify the membrane potential of neurons. The K^+ levels can be regulated by K^+ transport through glial cells (Orkand et al., 1966). This can occur through spatial buffering that is, transfer of the extracellular K^+ from regions where K^+ is higher to distant regions where it is lower (Orkand et al., 1966), or by local accumulation of K^+ in glial cells (Barres et al., 1988, 1990). Glial cells can also help to regulate ion concentrations in synaptic spaces and thereby regulate synaptic transmission. For example, Ca^{2+} accumulation by glia would lower Ca^{2+} in the synaptic cleft, thus reducing Ca^{2+} -dependent transmitter release (Kuffler 1967; Barres 1991). Changes in pH can affect neural behavior and excitability (Balestrino and Somjen, 1988; Tang et al., 1990). In the optic nerve, an increase in the extracellular K^+ during optic discharge, can depolarize astrocytes and progressively alkalinize them due to enhanced $Na^+-HCO_3^-$ co-transport, a pH regulating mechanism present in glia but not in neurons (Grichtchenko and Chesler, 1994; Kettenmann and Schlue, 1988; Pappas and Ransom, 1994; Ransom and Orkand 1994). The alkaline shift in astrocytes leads to an extracellular acid shift (Ransom, 1992). Since small acid shifts can reduce neural excitability (Balestrino and Somjen, 1988; Tang et al., 1990), this represents a feedback mechanism by glia to regulate neural activity. Also, glial cells participate in the clearance of neurotransmitters from the synaptic cleft through transporter proteins, thereby contributing to the regulation of the duration of the synaptic signal (Mennerick and Zorumsky, 1994; Rothstein et al., 1996; Bergles and Jahr, 1997; Eliasof et al., 1998). For example they take up the excitatory neurotransmitter, glutamate through glutamate transporters (Roseth et al., 1995; Rothstein et al., 1996; Bergles and Jahr, 1997). Thus, they reduce glutamate receptor desensitization in neurons and also protect neurons from excitotoxicity by removing excess glutamate from the synaptic space.

Metabolic support to neurons: Glial cells are involved in the formation of the blood-brain barrier. They have been suggested to regulate blood flow to neurons through the microcirculation by mediating the local increases in the blood flow after regional increases in neuronal activity (Newman et al., 1984; Barres 1991, Clark and Mobbs, 1992). Since the

same glial cell can contact both axons and blood vessels (Peters et al., 1976; Suarez and Raff, 1989), a neuron to glia to blood vessel signaling mechanism could operate to regulate blood flow. Also, it has been suggested that astrocytes, the only cells in the CNS that contain significant amounts of glycogen (Coles, 1995; Poitry-Yamate et al., 1995; Tsacopoulos and Magistretti, 1996), are closely attuned to metabolic needs of neurons and release energy, in the form of lactate, upon demands of neuronal consumption.

Neuronal development; survival and differentiation: Anatomical studies have long since indicated that glia may function as substrate pathways during the CNS development (Schemchel and Rakic, 1979, Levitt and Rakic 1980). Glial cells are now found to be required for the formation and maintenance of axonal pathways by forming a scaffold that promotes axon extension and guides growth cones of pioneer neurons (Noble et al., 1984; Bastiani and Goodman, 1986; Kuwada, 1986; Jacobs and Goodman, 1989; Hosoya et al., 1995; Jones et al., 1995; Pfrieger and Barres, 1995), and for fasciculation, the process of bundling of individual growing axons into fascicles (Silver and Shapiro, 1981; Noble and Cohen, 1984; Hosoya et al., 1995; Jones et al., 1995; Pfrieger and Barres, 1995). In vertebrates, cell adhesion molecules such as neuronal cell adhesion molecule (N-CAM), are expressed on the surface of the glial cells and appear to be involved in growth cone guidance (Edelman, 1983; Noble et al., 1985; Rutishauser, 1984).

Additionally, glia release a number of soluble peptides such as, glia derived neurotrophic factor (GDNF) and BDNF (brain derived nerve factor), which promote dendritic and axonal development (Dennis-Donini et al., 1984; Chamak et al., 1987; Rousset et al., 1988; Johnson et al., 1989; Tropea et al., 1989; Lin et al., 1993). These neurotrophins also promote neuronal survival and differentiation (Barde, 1989; Engele and Church, 1991; Engele et al., 1991; Gaul and Lubbert, 1992; Lin et al., 1993; Raff et al., 1993; Takeshima et al., 1994; Pfrieger and Barres, 1995; Kaplan et al., 1997). Furthermore, it was recently shown that synaptic efficacy is enhanced by glial cells in vitro (Pfrieger and Barres, 1997). In glia-free cultures synapses formed with normal ultrastructure but displayed little spontaneous activity and high failure rates in synaptic transmission. In cocultures with glia, the frequency and amplitude of spontaneous postsynaptic currents

were potentiated and fewer transmission failures occurred, without affecting neuronal survival. These studies suggest that developing neurons in culture form inefficient synapses that require glial signals to become fully functional.

Astrocyte-astrocyte communication occurs through variations in intracellular Ca^{2+} that propagate as Ca^{2+} waves

Glial cells make up almost one half of the brain volume and far outnumber neurons. Although their importance in neuronal development and upkeep was acknowledged, until recently it was thought that glia do not have a direct role in the active intercellular signaling in the CNS. that they are mere bystanders in the signaling processes underlying information encoding and processing. Relegation of glia to a secondary role has occurred mainly for the reason that only neurons exhibit electrical excitability which is an important characteristic required for signal encoding and processing. However, astrocytes have now been shown to exhibit a form of excitability and intercellular communication using variations in their cytosolic Ca^{2+} concentrations. A number of stimuli, mechanical, electrical or application of ligands such as glutamate, norepinephrine, ATP or prostaglandins, to astrocytes, can lead to elevations in their internal Ca^{2+} which can then propagate through the network of neighboring astrocytes as a wave of increase in free Ca^{2+} (Cornell-Bell et al., 1990; Charles et al., 1991; Duffy and MacVicar, 1995; Salter and Hicks, 1995; Porter and McCarthy, 1996; Verkhratsky and Kettenmann, 1996; Newman and Zahs, 1997; Araque et al, 1998a,b; Bezzi et al., 1998).

The precise mechanisms governing initiation and propagation of Ca^{2+} wave have not been clearly defined yet. One model suggests that diffusion of the second messenger IP_3 through functional gap junctions between astrocytes, mediates the intercellular Ca^{2+} waves (Finkbeiner 1992; Berridge 1993; Charles 1993; Sneyd et al., 1995; Horne and Meyer, 1997; Venance et al. 1997) by mobilization of IP_3 sensitive internal Ca^{2+} stores. Consistent with this model, gap junction inhibitors, halothane and octanol block propagation of the Ca^{2+} wave (Finkbeiner, 1992; Giaume and McCarthy, 1996; Venance et al., 1997). Also,

thapsigargin, an inhibitor of the Ca^{2+} -ATPase of the endoplasmic reticulum, responsible for filling the IP_3 -sensitive internal Ca^{2+} stores, blocks the spread of the Ca^{2+} wave (Boitano et al., 1992; Charles et al., 1993; Araque et al., 1998a,b). Another model suggests that the propagation of Ca^{2+} wave may occur through Ca^{2+} -induced- Ca^{2+} -release, a regenerative interaction between Ca^{2+} and the release of additional Ca^{2+} from intracellular stores (Meyer, 1991; Charles et al., 1993; Wang et al., 1997).

In addition, extracellular signals could also be involved in the formation of Ca^{2+} waves since isolated astrocytes that had no physical contact with other astrocytes in culture, have been shown to participate in these waves (Hassinger et al., 1996a). In this interesting preparation, cell-free lanes ($\sim 100 \mu\text{m}$ wide) were created in confluent astrocyte cultures by deleting cells with a glass pipette. Electrical stimulation of astrocytes initiated Ca^{2+} waves which crossed over these lanes. Furthermore, local perfusion of the stimulus site altered the direction and size of the wave, suggesting that the perfusion affected the diffusion of an agent released from the astrocytes thereby altering the Ca^{2+} wave.

Neuronal activity can trigger astrocyte Ca^{2+} waves

Astrocytes possess several ion channels including voltage-gated Na^+ and K^+ channels as well as receptors for various neurotransmitters such as glutamate, norepinephrine, serotonin, histamine (Barres et al., 1990; Cornell-Bell et al. 1990; Duffy and MacVicar 1995; Salter and Hicks 1995; Porter and McCarthy 1996). Exposure to these neurotransmitters induced propagating Ca^{2+} waves in cultured astrocytes (Ahmed et al., 1990; Inagaki et al., 1991; Glaum et al., 1990; Sugino et al., 1984). Recently, it was shown that neuronal activity and synaptic release of the neurotransmitter glutamate, can trigger Ca^{2+} waves among astrocytes in hippocampal slice cultures as well as in acutely isolated hippocampal slices (Dani et al. 1992; Porter and McCarthy 1996, Pasti et al. 1997). These observations raised the exciting possibility that if these neuronally-induced astrocyte Ca^{2+} signals were to somehow influence neuronal excitability or synaptic currents, astrocytes could participate in bidirectional communication with neurons.

Astrocytes release the excitatory neurotransmitter, glutamate

Astrocytes release to the extracellular space many substances such as metabolites or growth factors that are essential for neuronal function (Barres, 1991; Pfriger and Barres, 1995; Tsacopoulos and Magistretti, 1996). In addition, cultured astrocytes have also been shown to release glutamate, the most abundant excitatory transmitter in the CNS (Szatkowski et al., 1990; Kimelberg et al., 1990; Parpura et al., 1994; Parpura et al., 1995; Pocock et al., 1995; Bezzi et al., 1998).

At least three different putative mechanisms of release have been proposed:

Reversal of glutamate transporters: Glutamate uptake in glial cells functions to remove excess glutamate from the extracellular space in the CNS and occurs through glutamate transporters (Hertz 1979). Glutamate uptake is coupled with outward transport of K^+ (antiport) and inward transport of Na^+ ions (symport). Accordingly, it is activated by intracellular K^+ and extracellular Na^+ and is associated with a net inward current. Szatkowski et al. (1990) reported that in Müller cells, a type of retinal glial cells, raising extracellular K^+ concentration evokes glutamate release. This glutamate release was associated with an outward current and was activated by intracellular Na^+ and extracellular K^+ . On the other hand the release was inhibited by extracellular Na^+ and intracellular K^+ . Taken together these observations show that the glutamate release is occurring due to a counter transport of ions, indicating that it was through the reversal of the glutamate transporter. The glutamate uptake system in Müller cells has properties similar to that of the mammalian glial cells, including astrocytes, thus suggesting a Ca^{2+} -independent, transporter-mediated mechanism of glutamate release from astrocytes.

Swelling-induced release of glutamate: Astrocytes have been shown to release glutamate through a volume-sensitive mechanism. Exposure to hypotonic media leads to the release of glutamate from astrocytes (Pasantes-Morales and Schousboe, 1989; Pasantes-Morales et al., 1990, Kimelberg et al., 1990; Martin et al., 1990; O'Connor and Kimelberg, 1993). This swelling-induced release is Ca^{2+} -independent since it is unaffected by removal of extracellular Ca^{2+} or by buffering of intracellular Ca^{2+} .

Ca²⁺-dependent release of glutamate: Parpura et al. (1994) demonstrated that application of the peptide bradykinin increases the intracellular Ca²⁺ concentration in astrocytes which leads to the release of glutamate. When the external Ca²⁺ was removed, bradykinin neither induced a Ca²⁺ elevation in astrocytes nor glutamate release from them, indicating a requirement for Ca²⁺ in glutamate release. Addition of the Ca²⁺ ionophore ionomycin, in the presence of external Ca²⁺, but not in its absence, stimulated glutamate release from astrocytes. Thus, an increase in Ca²⁺ was shown to be necessary and sufficient for the release of glutamate. Furthermore, this release was seen to be insensitive to glutamate transporter inhibitors and was not mediated by cell swelling (1994, 1995a). This Ca²⁺-dependence of the glutamate release from astrocytes has been confirmed by a number of recent studies (Araque et al., 1998a,b, Bezzi et al., 1998).

In addition to its Ca²⁺-dependence, glutamate release from astrocytes has been shown to be sensitive to α -latrotoxin (Parpura et al., 1995a), a toxin from the black widow spider venom that induces neuronal synaptic transmitter release by evoking massive vesicle exocytosis and eventually, depletes the vesicle pool. The sensitivity of glutamate release to the α -latrotoxin and the expression in astrocytes of a number of synaptic proteins involved in neuronal synaptic vesicle release, such as synaptobrevin and syntaxin (Parpura et al., 1995b), have raised the exciting possibility that astrocytes may release glutamate through a Ca²⁺-dependent vesicular exocytosis mechanism, resembling the neuronal transmitter release mechanism. Consistent with this idea, Bezzi et al. (1998) have recently provided evidence that tetanus toxin, which blocks neuronal synaptic transmitter release by specifically cleaving the synaptic vesicle integral protein synaptobrevin, also blocks glutamate release from astrocytes. Since synaptobrevin is also expressed by astrocytes, it suggests that a functional synaptobrevin is required for the Ca²⁺-dependent glutamate release from astrocytes, and therefore, that vesicular exocytosis mediates glutamate release. However, ultrastructural data providing a clear demonstration of the presence of vesicles within astrocytes responsible for this release pathway is still lacking. Nonetheless, current data supports the existence of a Ca²⁺-dependent vesicular exocytotic mechanism of glutamate release from astrocytes.

Glutamate is the major excitatory neurotransmitter in the nervous system

Glutamate, the most abundant excitatory neurotransmitter in the CNS, is believed to have important roles in neuronal plasticity, cognition, memory, learning and some neurological disorders such as epilepsy, stroke and neurodegeneration (for reviews see Choi, 1988; Baskys, 1992; Nakanishi et al., 1992; Schoepp and Conn, 1993). Glutamate mediates its action through two distinct classes of receptors, termed ionotropic, which are directly coupled to intrinsic ion channels, and metabotropic, which are coupled to ion channels indirectly through second messenger systems.

The ionotropic receptors are further subdivided into two subtypes, depending on their affinity for the amino acid analog N-methyl-D-aspartate (NMDA).

NMDA receptors: The NMDA receptor is coupled to a cation channel that is permeable to Na^+ , K^+ and Ca^{2+} . This channel exhibits a unique property of being gated by both glutamate and voltage. At resting membrane potential (-60 mV) the channel is blocked by Mg^{2+} from the extracellular space. In this state, even in the presence of glutamate the channel remains closed due to the Mg^{2+} block. However, when the membrane is depolarized in the presence of glutamate, the voltage-dependent Mg^{2+} block is expelled and the channel is opened so that Na^+ and Ca^{2+} can enter the cell. Thus, the channel permits an ion flux when both the membrane is depolarized and glutamate is present. Removal of Mg^{2+} in the presence of glycine, a co-agonist, achieves maximal activation of the NMDA channel. The Ca^{2+} entry through the NMDA-activated channel further activates Ca^{2+} -dependent second messenger cascades. NMDA receptors have been implicated in the mechanisms underlying memory and learning (Madison et al., 1991; Malgaroli and Tsien, 1992; Manabe et al., 1992; Bliss and collingridge, 1993).

non-NMDA receptors: These are further subdivided into AMPA receptors and kainate receptors since they show high affinity for the selective agonist AMPA (D,L-a-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid) and kainate respectively. These receptors contain an intrinsic ion channel enabling the movement of Na^+ and K^+ ions. Activation of non-NMDA receptors results in the movement of Na^+ and K^+ ions and causes depolarization of the membrane.

The metabotropic glutamate receptors can be divided into eight subtypes based on sequence analysis; *mGluR₁*, *mGluR₂*, *mGluR₃*, *mGluR₄*, *mGluR₅*, *mGluR₆*, *mGluR₇* and *mGluR₈* (Gereau and Conn, 1995). All of these are integral membrane proteins and contain characteristic seven hydrophobic domains, which are thought to represent the transmembrane spanning region. These receptors are coupled to GTP binding proteins (G-proteins) and activation of these receptors leads to G-protein mediated second messenger pathways (Gilman, 1987; Levitan and Kaczmarek, 1991; Watson and Arkininstall, 1994; Gereau and Conn, 1995).

The mGluRs can be grouped into three major classes depending upon their signal transduction pathways. Class I receptors which include *mGluR₁* and *mGluR₅*, activate the phosphoinositide pathway through a G-protein of the G_q/G_{11} class. Class II receptors which include *mGluR₂*, *mGluR₃* and *mGluR₄* and class III which include *mGluR₆*, *mGluR₇* and *mGluR₈*, inhibit adenylyl cyclase most likely through the G_i/G_o class.

Phosphoinositide-specific phospholipase C (PLC) plays a pivotal role in the signal transduction cascade involving the class I receptors (*mGluR₁* and *mGluR₅*). Glutamate-dependent activation of the *mGluR₁* and *mGluR₅* leads to G-protein mediated activation of PLC which hydrolyses the membrane phospholipid, phosphatidylinositol 4,5-biphosphate (PIP_2) with consequent generation of the two second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses into the cytoplasm and binds to specific receptors on the endoplasmic reticulum. This binding opens the Ca^{2+} channels in the membrane, as a result of which Ca^{2+} ions are released into the cytoplasm from the intracellular Ca^{2+} storage sites in the endoplasmic reticulum. The elevated cytoplasmic Ca^{2+} can influence a myriad of Ca^{2+} -dependent enzymes such as protein kinase C (PKC) and Ca^{2+} /calmodulin dependent protein kinase II, and mediate diverse cellular functions. The other second messenger, DAG, acts as a substrate for the enzyme phospholipase A_2 (PLA_2) and is broken down into arachidonic acid (AA). AA can be metabolized via several different enzymes to yield biologically active products. For example, the enzyme cyclooxygenase can metabolize AA to form prostaglandins, which as we will see in Chapter 4, can have important implications for astrocyte-neuron interactions. Both DAG and AA can also activate PKC.

The mGluR₂, mGluR₃ and mGluR₄ inhibit adenylyl cyclase most likely through the G_i/G_o class. The enzyme adenylyl cyclase synthesizes cyclic AMP (cAMP) from ATP. The second messenger cAMP activates the enzyme, cAMP-dependent protein kinase (PKA) which can regulate the activity of a number of effector proteins by phosphorylation.

Astrocytes can signal to neurons by releasing glutamate

Since neuronal activity can trigger Ca²⁺ elevations in astrocytes (Dani et al. 1992; Porter and McCarthy 1996; Pasti et al. 1997) which can then induce the Ca²⁺-dependent glutamate release from astrocytes (Szatkowski et al., 1990; Kimelberg et al., 1990; Parpura et al., 1994; Parpura et al., 1995; Bezzi et al., 1998; Hassinger et al., 1995), the next obvious question is, can this glutamate released from astrocytes in turn, evoke a feedback signal to neurons? The first evidence for the glutamate-mediated astrocyte to neuron signaling came from four groups (Parpura et al., 1994; Nedergaard, 1994; Charles, 1994; Hassinger et al., 1995). They showed that a variety of stimuli (application of the neuropeptide bradykinin, photostimulation, mechanical and electrical stimulation) which raised the internal Ca²⁺ in the astrocytes, evoked glutamate release from astrocytes which led to further Ca²⁺ elevations in adjacent neurons. These observations were subsequently attested to by several different studies in cell culture and *in situ* (Hassinger et al., 1995; Pasti et al., 1997; Bezzi et al., 1998; Newman and Zahs, 1998). Pharmacological studies showed that the astrocyte-induced Ca²⁺ elevations in neurons were inhibited by glutamate receptor antagonists. Parpura et al. (1994) reported that the NMDA glutamate receptors were involved in mediating this response, while other groups provided evidence implicating both NMDA and non-NMDA receptors (Hassinger et al., 1995; Pasti et al., 1997; Bezzi et al., 1998). Although most studies confirm that the astrocyte-induced neuronal Ca²⁺ elevations are mediated through glutamate released from astrocytes, Nedergaard (1994) reported that this signaling was attenuated by gap junction blockers suggesting the existence of an additional pathway involving gap junctions.

The findings that astrocytes communicate with each other through variations in their Ca²⁺ concentrations, that neuronal activity can trigger these Ca²⁺ changes in astrocytes, and

finally, that Ca^{2+} elevations in astrocytes can evoke Ca^{2+} increases in adjacent neurons, have raised the possibility that astrocytes and neurons may exist as a network in which bidirectional communication takes place. Although the existence of communication between neurons and astrocytes is firmly established by Ca^{2+} imaging studies, the physiological consequences of such communication are yet to be elucidated. Can the astrocyte-neuron signaling be detected electrophysiologically? Can astrocytes alter neuronal physiology, and modulate neuronal currents and synaptic transmission? These questions are the topic of investigation in this dissertation.

Chapters 2, 3 and 4 show that stimulation of astrocytes either mechanically, electrically or by application of a neuroligand, prostaglandin E_2 , gives rise to an intercellular Ca^{2+} wave among astrocytes which then evokes a slow inward current (SIC) in adjacent neurons and/or increases the frequency of the miniature post synaptic currents (mPSCs). This astrocyte-induced modulation of neuronal currents requires a Ca^{2+} elevation and the presence of a Ca^{2+} wave among astrocytes. The SIC is mediated through NMDA and non-NMDA glutamate receptors while the increase in the mPSCs occurs through activation of the NMDA receptors. In addition, stimulation of astrocytes reduces action potential-evoked postsynaptic currents through the activation of the mGlu Receptors. The results presented in this dissertation clearly demonstrate that astrocytes modulate neuronal currents and synaptic transmission and provide direct evidence for astrocyte-neuron signaling.

Dissertation organization

The dissertation contains a total of five chapters which include three separate manuscripts as three chapters. The two additional chapters are the "general introduction" and "general conclusions".

Chapter 1 titled "general introduction" provides the background and significance of the work presented in this dissertation and serves as a common introduction for the three different manuscripts. It is divided into three subsections; introduction which includes a literature review, dissertation organization and references.

Chapter 2, 3 and 4 are three independent manuscripts prepared for three different journals. Each chapter follows the style and subsection format as per the requirements of the journal, and includes abstract, introduction, materials and methods, results, discussion, acknowledgments, references, figures and figure legends. References are listed at the end of the chapter in the style used by the corresponding journal. Figures are presented together after the references with each figure number clearly shown beneath, followed by the figure legends. Since these manuscripts have multiple authors, each chapter begins with a statement about my specific contribution to the paper.

Chapter 2 titled "Glutamate-Dependent Astrocyte Modulation Of Synaptic Transmission Between Cultured Hippocampal Neurons" is published in the European Journal of Neuroscience.

Chapter 3 titled "Calcium Elevation In Astrocytes Causes An NMDA Receptor-Dependent Increase In The Frequency Of Miniature Synaptic Currents In Cultured Hippocampal Neurons" is In Press in the Journal of Neuroscience.

Chapter 4 titled "Prostaglandin E_2 Evokes Glutamate-Mediated Astrocyte-Neuron Signaling in Cultured Hippocampal Cells" is a manuscript submitted for publication to the Journal of Neuroscience.

The experiments performed in these chapters are of a collaborative nature and require using a multidisciplinary approach. Accordingly, the papers are credited to multiple authors. The bulk of the studies reported in the European Journal of Neuroscience (Chapter 2) and the Journal of Neuroscience (Chapter 3), were carried out by A. Araque, who is the first author on these papers. However, my contribution was key to the conclusions reached, without which these papers could not be published. Therefore, I am included as a middle author (third and second respectively) on these papers. I am the first author on Chapter 4, since I am the major contributor to this work.

Chapter 5 titled "general conclusions" summarizes the results presented in chapters 2, 3 and 4 and contains a discussion of the results as they apply to the larger question detailed in the general introduction.

References

- Adrian, E. D. 1946 The physical background of perception. Clarendon Press, Oxford.
- Ahmed, Z., Lewis, C.A., and Faber, D.S. 1990. Glutamate stimulates release of Ca^{2+} from internal stores in astroglia. *Brain Res.* **516**: 165-169.
- Araque, A., Parpura, V., Sanzgiri, R.P., and Haydon, P.G. 1998a. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur. J. Neurosci.* **10**: 2129-2142.
- Araque, A., Sanzgiri, R.P., Parpura, V., and Haydon, P.G. 1998b. Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J. Neurosci.* (in press).
- Balestrino, M., and Somjen, G.G. 1988. Concentration of carbon dioxide, interstitial pH and synaptic transmission in hippocampal formation of the rat. *J. Physiol. Lond.* **396**: 247-266.
- Banker, G. 1980. Trophic interactions between astroglial cells and hippocampal neurons in culture. *Science* **209**: 809-810.
- Barres, B. A., Chun, L.L.Y., and Corey, D.P. 1988 Ion channel expression by white matter glia: Type 2 astrocytes and oligodendrocytes. *Glia* **1**: 10-30.
- Barres, B.A., Koreoshetz, W.J., Chun, L.L.Y., and Corey, D.P. 1990 Ion channel expression by white matter glia: the type I astrocyte. *Neuron* **5**: 527-544.
- Barres, B.A. 1991 New roles for glia. *J. Neurosci.* **11**: 3685-3694.
- Baskys, A. 1992. Metabotropic receptors and slow excitatory actions of glutamate agonists in the hippocampus. *Trends Neurosci.* **15**: 92-96.
- Baskys, A. and Malenka, R.C. (1991) Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. *J. Physiol. Lond.*, **444**, 687-701.
- Bastiani, M.J., and Goodman, C.S. 1986. Guidance of neuronal growth cones in the Grasshopper embryo. III. Recognition of specific glial pathways. *J. Neurosci.* **6**: 3542-3551.

- Bergles, D.E., and Jahr, C.E. 1997. Synaptic activation of glutamate transporters in hippocampal astrocytes. *Neuron* **19**: 1297-1308.
- Berridge, M.J., and Galione, A. 1988. Cytosolic calcium oscillations. *FASEB J.* **2**: 3074-3082.
- Berridge, M. J. 1993. Inositol triphosphate and calcium signaling *Nature* **361**: 315-325.
- Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Lodi Rizzini, B., Pozzan, T., and Volterra, A. 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* **391**: 281-285.
- Bliss, T.V.P., and Collingridge, G.L. 1993. A synaptic model of memory: long term potentiation in the hippocampus. *Nature* **361**: 31-39.
- Boitano, S., Dirksen, E.R., and Sanderson, M.J. 1992. Intercellular propagation of calcium waves mediated by inositol triphosphate. *Science* **258**: 292-295.
- Chamak B., Fellous, A., Glowinski J., and Prochiantz, A. 1987 MAP2 expression and neuritic outgrowth and branching are coregulated through region-specific neuro-astroglial interactions. *J. Neurosci.* **7**: 3163-3170.
- Charles, A.C. 1994. Glia-neuron intercellular calcium signaling. *Dev. Neurosci.* **16**: 196-206.
- Charles, A.C., Dirksen, E.R., Merrill, J.E., and Sanderson, M.J. 1993. Mechanisms of intercellular calcium signaling in glial cells studied with dantrolene and thapsigargin. *Glia* **7**: 134-145.
- Charles, A.C., Merrill, J.E., Dirksen, E.R., and Sanderson, M.J. 1991. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* **6**: 983-992.
- Clark, B., and Mobbs, P. 1992. Transmitter opened channels in retinal astrocytes studied in vivo by whole cell patch-clamping. *J. Neurosci.*
- Coles, J.A. 1995. *Neuroglia* pp. 793-804, Oxford University Press.
- Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., and Smith, S.J. 1990. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* **247**: 470-473.

- Dani, J.W., Chernjavsky, A., and Smith, S.J. 1992. Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* **8**: 429-440.
- Dennis-Donini S., Glowinski J., and Prochiantz, A. 1984 Glial heterogeneity may define the three-dimensional shape of the mouse mesencephalic dopaminergic neurones. *Nature* **307**: 641-643.
- Duffy, S., and MacVicar, B.A. 1995. Adrenergic calcium signaling in astrocyte networks within the hippocampal slice. *J. Neurosci.* **15**: 5535-5550.
- Edelman, G. M. 1983. Cell adhesion molecules. *Science* **219**: 450-457.
- Eliasof, S., Arriza, J.L., Leighton, B.H., Kavanaugh, M.P., and Amara, S.G. 1998. Excitatory amino acid transporters of the salamander retina: Identification, localization, and function. *J. Neurosci.* **18**: 698-712.
- Engel, J., and Church Hill, B. 1991. The neurotrophic effect of fibroblast growth factors on dopaminergic neurons in vitro are mediated by mesencephalic glia. *J. Neurosci.* **11**: 3070-3078.
- Engel, J., Schubert, D., and Bohn, M.C. 1991. Conditioned media derived from glial cell lines promote survival and differentiation of dopaminergic neurons in vitro: Role of mesencephalic glia. *J Neurosci. Res.* **30**: 359-371.
- Finkbeiner, S.M. 1992. Calcium waves in astrocytes - Filling in the gaps. *Neuron* **8**: 1101-1108.
- Gaul G., and Lübbert, H. 1992. Cortical astrocytes activated by basic fibroblast growth factor secrete molecules that stimulate differentiation of mesencephalic dopaminergic neurons. *Proc. R. Soc. Lond. B.* **249**: 57-63.
- Gereau, R.W., and Conn, P.J. 1995. Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. *J. Neurosci.* **15**, 6879-6889.
- Giaume, C., and McCarthy, K.D. 1996. Control of gap-junctional communication in astrocytic networks. *Trends Neurosci.* **19**: 319-325.
- Gilman, A.G. 1987. G proteins: Transducers of receptor generated signals. *Annu. Rev. Biochem.* **56**: 615-649.

- Glaum, S.R., Holzwarth, J.A., and Miller, R.J. 1990. Glutamate receptors activate Ca^{2+} mobilization and Ca^{2+} influx into astrocytes. *Proc. Natl. Acad. Sci. U.S.A.* **87**: 3454-3458.
- Grichtchenko, I.I., and Chesler, M. 1994. Depolarization-induced alkalization of astrocytes in gliotic hippocampal slices. *Neuroscience* **62**: 1057-1070.
- Hassinger, T.D., Atkinson, P.B., Strecker, G.J., Whalen, L.R., Dudek, F.E., Koseel, A.H., and Kater, S.B. 1995. Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. *J. Neurobiol.* **28**: 159-170.
- Hassinger T.D., Guthrie, P.B., Atkinson, P.B., Bennet, M.V.L., and Kater, S.B. 1996. An extracellular signaling component in propagation of astrocytic calcium waves. *Proc. Natl. Acad. Sci. USA* **93**: 13268-13273.
- Hertz, L. 1979. Functional interactions between neurons and astrocytes I. Turnover and metabolism of putative amino acid transmitters. *Prog. Neurobiol.* **13**: 277-323.
- Hille, B. 1992. *Ionic Channels of Excitable Membranes*. 2nd ed., Sinauer Associates, Sunderland.
- Horne, J. H., and Meyer, T. 1997. Elementary calcium units induced by inositol triphosphate. *Science* **276**: 1690-1693.
- Hosoya, T., Takizawa, K., Nitta, K., and Hotta, Y. 1995. Glial cells missing: a binary switch between neuronal and glial determination in *Drosophila*. *Cell* **82**: 1025-1036.
- Inagaki, N. Fukui, H., Ito, S., Yamatodani, A., and Wada, H. 1991. Single type-2 astrocytes show multiple independent sites of Ca^{2+} signaling in response to histamine. *Proc. Natl. Acad. Sci. U.S.A.* **88**: 4215-4219.
- Jacobs, J. R., and Goodman, C.S. 1989. Embryonic development of axonal pathways in the *Drosophila* CNS. II. Behavior of pioneer growth cones. *J. Neurosci.* **9**: 2402-2422.
- Jones, B.W., Fetter, R.D., Tear, G., and Goodman, C.S. 1995. Glial cells missing: a genetic switch that controls glial versus neuronal fate. *Cell* **82**: 1013-1024.
- Johnson, M.I., Higgins, D., and Ard, M.D. 1989. Astrocytes induce dendritic development in cultured sympathetic neurons. *Dev. Brain Res.* **47**: 289-292.
- Kandel, E.R., Schwartz, J.H., and Jessell, T.M. 1995. *Essentials of neural science and behavior*. Appleton and Lange, Norwalk.

- Kao, C.T. 1966. Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmacol. Rev.* **18**: 977-1049.
- Kaplan, M.R., Meyer-Franke, A., Lambert, S., Bennett, V., Duncan I.D., Levinson, S.R., and Barres, B.A. 1997. Induction of sodium channel clustering by oligodendrocytes. *Nature*. **386**: 724-728.
- Kettenmann, H. 1996. Beyond the neuronal circuitry. *Trends Neurosci.* **19**: 305-306.
- Kettenmann, H. and Schlue, W.-R. 1988. Intracellular pH regulation in cultured mouse oligodendrocytes. *J. Physiol. Lond.* **406**: 147-162.
- Kimelberg, H.K., Goderie, S.K., Higman, S., Pang, S., and Waniewski, R.A. 1990. Swelling-induced release of glutamate, aspartate, and taurine from astrocytes cultures. *J. Neurosci.* **10**: 1583-1591.
- Kuffler, S.W. 1967. Neuroglial cells: physiological properties and a potassium mediated effect of neuronal activity on the glial membrane potential. *Proc. R. Soc. Lond. (Biol)* **168**: 1-21.
- Kuffler, S.W., Nicholls, J.G., and Martin, R.A. 1984. *From neuron to brain*. 2nd ed., Sinauer Associates, Sunderland.
- Kuwada, J.Y. 1986. Cell recognition by neuronal growth cones in a simple vertebrate embryo. *Science* **233**: 740-746.
- Largo, C., Cuevas, P., Somjen, G.G., Martin del Rio, R., and Herreras, O. 1996. The effect of depressing glial function in rat brain *in situ* on ion homeostasis, synaptic transmission, and neuron survival. *J. Neurosci.* **16**: 1219-1229.
- LeRoux, P.D., and Reh, T.A. 1994. Regional differences in glial derived factors that promote dendritic outgrowth from mouse cortical neurons in vitro. *J. Neurosci.* **14**: 4639-4655.
- Levitan, I.B., and Kaczmarek, L.K. 1991. *The neuron, cell and molecular biology* pp 233-240. Oxford University Press.
- Levitt, P., and Rakic, P. 1980. Immunoperoxidase localization of glial fibrillary acidic protein in radial cells and astrocytes of the developing rhesus monkey brain. *J. Comp. Neurol.* **193**: 815-840.

- Madison, D.V., Malenka, R.C., and Nicoll, R.A. 1991. Mechanisms underlying long-term potentiation of synaptic transmission. *Annu. Rev. Neurosci.* **14**: 379-397.
- Malgaroli, A., and Tsien, R.W. 1992. Glutamate-induced long term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neurons. *Nature* **357**: 134-139.
- Manabe, T., Pius, R., and Roger, A. N. 1992. Postsynaptic contribution to long-term potentiation revealed by the analysis of miniature synaptic currents. *Nature* **355**: 50-55.
- Martin, D.L., Madelian, V., Seligmann, B., and Shain, W. 1990. The role of osmotic pressure and membrane potential in K⁺-stimulated taurine release from cultured astrocytes and LRM55 cells. *J. Neurosci.* **10**: 571-577.
- Mennerick, S., and Zorumski, C.F. 1994. Glial contribution to excitatory neurotransmission in cultured hippocampal cells. *Nature* **368**: 59-62.
- Meyer, T. 1991. Cell signaling by second messenger waves. *cell* **64**: 675-678.
- Nakanishi, S. 1992. Molecular diversity of glutamate receptors and implications for brain function. *Science* **258**: 597-603.
- Nedergaard, M. 1994. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* **263**: 1768-1771.
- Newman, E.A., Frambach, D.A. and Odette, L.L. 1984. Control of extracellular potassium levels of retinal glial cell K siphoning. *Science* **225**: 1174-1175.
- Newman, E.A., and Zahs, K.R. 1997. Calcium waves in retinal glial cells. *Science* **275**: 844-847.
- Newman, E.A., and Zahs, K.R. 1998. Modulation of neuronal activity by glial cells in the retina. *J. Neurosci.* **18**: 4022-4028.
- Noble, M., Albrechtsen, M., Moller, C., Lyles, J., Bock, E., Gordis, C., Watanabe M., and Rutishauser U. 1985. Glial cells express N-CAM/D2-CAM-like polypeptides in vitro. *Nature* **316**: 725-728.
- Noble, M., Fok-Seang, J., and Cohen, J. 1984. Glia are a unique substrate for the *in vitro* growth of central nervous system neurons. *J. Neurosci.* **4**: 1892-1903.

- O'Connor, E.R., and Kimelberg, H.K. 1993. Role of calcium in astrocyte volume regulation and in the release of ions and amino acids. *J. Neurosci.* **13**: 2638-2650.
- O'Connor, V., Augustine, J.G., and Betz, H. 1994. Synaptic vesicle exocytosis: Molecules and models. *Cell* **76**: 785-787.
- Orkand, R.K., Nicholls, J.G., and Kuffler, S.W. 1966. Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. *J. Neurophysiol.* **29**: 788-806.
- Parpura, V., Basarsky, T.A., Liu, F., Jęftinija, K., Jęftinija, S., and Haydon, P.G. 1994. Glutamate-mediated astrocyte-neuron signalling. *Nature* **369**: 744-747.
- Parpura, V., Liu, F., Brethorst, S., Jęftinija, K., Jęftinija, S., and Haydon, P.G. 1995a. α -Latrotoxin stimulated glutamate release from cortical astrocytes in cell culture. *FEBS Lett.* **360**: 266-270.
- Parpura, V., Fang, Y., Basarsky, T.A., Jahn, R., and Haydon, P.G. 1995b. Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP-25 in cultured astrocytes. *FEBS Lett.* **377**: 489-492.
- Pasantes-Morales, H., Moran, J., and Schousboe, A. 1990. Volume sensitive release of taurine from cultured astrocytes: properties and mechanism. *Glia* **3**: 4275-432.
- Pasantes-Morales, H., and Schousboe, A. 1989. Release of taurine from astrocytes during potassium-evoked swelling. *Glia* **2**: 45-50.
- Pasti, L., Volterra, A., Pozzan, T., and Carmignoto, G. 1997. Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes *in situ*. *J. Neurosci.* **17**: 7817-7830.
- Peters, A., Palay, S. L., and Webster, H. F 1976. The fine structure of the nervous system: the neurons and supporting cells, pp 233-244. Philadelphia: Saunders.
- Pfriege, F.W., and Barres, B.A. 1995. What the fly's glia tell the fly's brain. *Cell* **83**: 671-674.
- Pfriege, F.W., and Barres, B.A. 1997. Synaptic efficacy enhanced by glial cells in vitro. *Science* **277**: 1684-1687.

- Pocock, J.M., Cousin, M.A., Parkin, J., and Nicholls, D.G. 1995. Glutamate exocytosis from cerebellar granule cells: the mechanism of a transition to an L-type Ca^{2+} channel coupling. *Neurosci.* **67**: 595-607.
- Poitry-Yamate, C.L., Potry, S. and Tsacopoulos, M. 1995. Lactate released by Müller glial cells is metabolized by photoreceptors from mammalian retina. *J. Neurosci.* **15**: 5179-5191.
- Porter, J.T., and McCarthy, K.D. 1996. Hippocampal astrocytes *in situ* respond to glutamate released from synaptic terminals. *J. Neurosci.* **16**: 5073-5081.
- Quandt, F.N., and MacVicar, B.A. 1986. Calcium activated potassium channels in cultured astrocytes. *Neurosci.* **19**: 29-41.
- Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y., and Jacobson, M.D. 1993. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**: 695-700.
- Rakic, P. 1990. Principles of neuronal cell migration. *Experientia* **46**: 882-891.
- Ramón y Cajal, S. 1911. *Histologie du système nerveux de l'homme et des vertèbres*. Maloine, Paris.
- Ramón y Cajal, S. 1913. Contribución al conocimiento de la neuroglía del cerebro humano. *Trab. Lab. Invest. Biol. Univ. Madrid* **18**: 225-315.
- Ransom, B. R. 1992. Glial modulation of neural excitability mediated by extracellular pH: a hypothesis. *Prog. Brain Res.* **94**: 37-46.
- Ransom, B.R., and Orkand, R.K. 1996. Glial-neuronal interactions in non-synaptic areas of the brain: studies in the optic nerve. *Trends Neurosci.* **19**: 352-358.
- Roseth, S., Fykse, E.M., and Fonnum, F. 1995. Uptake of L-Glutamate into rat brain synaptic vesicles: effect of inhibitors that bind specifically to the glutamate transporter. *J. Neurochem.* **65**: 96-103.
- Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kunc, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P., and Welty, D.F. 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* **16**: 675-686.

- Rousselet, A., Felter, L., Chamak, B., and Prochiantz, A. 1988. Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia. *Dev. Biol.* **129**: 495-504.
- Salter, M.W., and Hicks, J.L. 1995. ATP causes release of intracellular Ca^{2+} via the phospholipase C / IP_3 pathway in astrocytes from the dorsal spinal cord. *J. Neurosci.* **15**: 2961-2971.
- Schmechel, D.E. and Rakic, P. 1979. A golgi study of radial glial cells in developing monkey telencephalon: Morphogenesis and transformation into astrocytes. *Anat. Embryol.* **156**: 115-152.
- Schoepp, D.D. and Conn, P.J. 1993. Metabotropic glutamate receptors in brain function and pathology. *Trends Pharmacol. Sci.* **11**: 508-515.
- Sherrington, C.S. 1947 The integrative action of the nervous system. Yale University Press, New Haven.
- Sliver, J. and Sapiro, J. 1981. Axonal guidance during development of the optic nerve: the role of pigmented epithelia and other extrinsic factors. *J. Comp. Neurol.* **202**: 521-538.
- Smith, G.M., Rutishouser, Y., Siver, J., and Miller R.H. 1990. Maturation of astrocytes *in vitro* alters the extent and molecular basis of neurite outgrowth. *Dev. Biol.* **138**: 377-390.
- Sneyd, J., Wetton, B.T.R., Charles, A.C., and Sanderson, M. J. 1995. Intercellular calcium waves mediated by diffusion of inositol triphosphate: a two dimensional model. *Am. J. Physiol.* **268**: 1537-1545.
- Sugino, H., Ogura, A., Kudo, Y. and Amano, T. 1984. Intracellular Ca^{2+} elevations induced by a neurotransmitter in a glial cell clone. *Brain Res.* **322**: 127-130.
- Suarez, I., and Raff, M.C. 1989. Subpial and pervalicular astrocytes associated with nodes of Ranvier in the rat optic nerve. *J. Neurocytol.* **18**: 577-582.
- Südhof, T. 1995. The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375**: 645-653.
- Szatkowski, M., Barbour, B., and Attwell, D. 1990. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* **348**: 443-447.

- Takeshima, T., Shimoda, K., Sauves, Y., and Commissiong, J.W. 1994. Astrocyte-dependent and -independent phases of the development and survival of rat embryonic day 14 mesencephalic, dopaminergic neurons in culture. *Neuroscience* **60**: 809-823.
- Tang, C.M., Dichter, M., and Morad, M. 1990. Modulation of the N-methyl-D-aspartate channel by extracellular H^+ . *Proc. Natl. Acad. Sci. U.S.A.* **87**: 6445-6449.
- Trimble, W.S., Linial, M., and Scheller, R.H. 1991. Cellular and molecular biology of the presynaptic nerve terminal. *Annu. Rev. Neurosci.* **14**: 93-122.
- Tropea, M., Johnson, M.I., and Higgins, D. 1989 Glial cells promote dendritic development in rat sympathetic neurons in vitro. *Glia* **1**: 380-392.
- Tsacopoulos, M., and Magistretti, P.J. 1996. Metabolic coupling between glia and neurons. *J. Neurosci.* **16**: 877-885.
- Virchow, R. 1846. Über das granulierte ansehn der wandungen der gerhirnventrikel. *Allg. Z. Psychiatr.* **3**: 242.
- Venance, L., Stella, N., Glowinski, J., and Giaume, C. 1997. Mechanism involved in initiation and propagation of receptor-induced intercellular calcium signaling in cultured rat astrocytes. *J. Neurosci.* **17**: 1981-1992.
- Verkhratsky, A., and Kettenmann, H. 1996. Calcium signalling in glial cells. *Trends Neurosci.* **19**: 346-352.
- Walz, W. 1989. Role of glial cells in the regulation of the brain ion microenvironment. *Prog. Neurobiol.* **33**: 309-333.
- Watson S., and Arkinstall, S. 1994. The G-protein linked receptor facts book. pp 130-141. Academic Press, New York.

CHAPTER 2. GLUTAMATE-DEPENDENT ASTROCYTE MODULATION OF SYNAPTIC TRANSMISSION BETWEEN CULTURED HIPPOCAMPAL NEURONS

A paper published in the European Journal of Neuroscience

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My contribution is involved in testing the hypothesis that a Ca^{2+} elevation, specifically in astrocytes, is necessary for the astrocyte-induced neuronal SIC. For this, I developed the following experimental approach. I micro-injected the Ca^{2+} chelator BAPTA into single astrocytes, along with the fluorescent dye fluoro-ruby to label the injected cell. Ca^{2+} changes in astrocytes were monitored by loading the cells with the Ca^{2+} indicator fluo-3. Mechanical stimulation of an uninjected astrocyte, as well as an astrocyte injected with fluoro-ruby alone, resulted in a Ca^{2+} elevation in the injected cell followed by an interastrocyte Ca^{2+} wave. In contrast, mechanical stimulation of an astrocyte injected with BAPTA together with fluoro-ruby, did not evoke Ca^{2+} elevations. Thus, I determined that BAPTA, not micro-injection *per se*, prevented Ca^{2+} elevations in the injected cell and the intercellular Ca^{2+} waves in response to mechanical stimuli. I also performed the micro-injections required for examining the effect of BAPTA on the neuronal SIC. My work is summarized in the figure 4.

ABSTRACT

The idea that astrocytes merely provide structural and trophic support for neurons has been challenged by the demonstration that astrocytes can regulate neuronal calcium levels. However, the physiological consequences of astrocyte-neuron signaling are unknown. Using mixed cultures of rat hippocampal astrocytes and neurons we have determined functional consequences of elevating astrocyte calcium levels on co-cultured neurons.

Electrical or mechanical stimulation of astrocytes to increase their calcium level, caused a glutamate-dependent slow inward current (SIC) in associated neurons. Microinjection of BAPTA into astrocytes to prevent the stimulus dependent increase in astrocyte calcium level, blocks the appearance of the neuronal SIC. Pharmacological manipulations indicate that this astrocyte-dependent SIC is mediated by extracellular glutamate acting on N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors. Additionally, stimulation of astrocytes reduced the magnitude of action potential evoked excitatory and inhibitory postsynaptic currents through the activation of metabotropic glutamate receptors. The demonstration that astrocytes modulate neuronal currents and synaptic transmission raises the possibility that astrocytes play a neuromodulatory role by controlling the extracellular level of glutamate.

INTRODUCTION

The role of astrocytes in the nervous system has been traditionally considered to be restricted to structural and trophic support for neurons. However, several observations suggest that astrocytes may have a more active role in the nervous system. Indeed, astrocytes exhibit a form of excitability and communication based on intracellular Ca^{2+} variations that can result in propagating intercellular Ca^{2+} waves (Cornell-Bell et al., 1990; Charles et al., 1991; Finkbeiner, 1992; Dani et al., 1992). Astrocytes express several ligand-gated ion channels that can trigger these Ca^{2+} waves (Cornell-Bell et al., 1990; Jensen and Chiu, 1990; Charles et al., 1991; Dani et al., 1992; Murphy et al., 1993; Kriegler and Chiu, 1993; Duffy and MacVicar, 1995). Neuronal activity may induce astrocyte Ca^{2+} waves *in vitro* (Dani et al., 1992) and *in situ* (Porter and MacCarthy, 1996), indicating that neurons can signal to astrocytes. Finally, astrocytes may in turn signal to neurons, since astrocyte stimulation can evoke neuronal calcium elevations (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995).

Recent studies have demonstrated, using Ca^{2+} imaging techniques, that either mechanical or electrical stimulation of astrocytes can induce a wave of elevated calcium in astrocytes and cause a delayed calcium signal in adjacent neurons (Nedergaard, 1994;

Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995). We now extend these earlier studies by determining the consequences of astrocyte stimulation on electrophysiological properties of hippocampal neurons and by asking whether this astrocyte-neuron signaling regulates synaptic transmission.

We demonstrate that astrocyte stimulation induces a long-lasting, slow inward current mediated by the activation of both NMDA and non-NMDA glutamate receptors. Since this current was insensitive to bath applied tetanus toxin, which prevents neuronal exocytosis, the glutamate must be directly released from astrocytes. Finally, we show that astrocyte stimulation reduces both excitatory and inhibitory synaptic transmission through the activation of selective presynaptic metabotropic glutamate receptors (mGluRs).

MATERIALS AND METHODS

Culture preparation

Primary cultures of mixed hippocampal neurons and astrocytes from 1- to 3-day-old postnatal rats were prepared as previously described (Basarsky et al., 1994) and were used after 8-20 days in culture. Enriched type-1 astrocytes were prepared as previously described (Parpura et al., 1995a). Cells were initially plated into culture flasks for 8-10 days, and then, following purification, astrocytes were replated onto glass coverslips and used in experiments after 3-4 days. Purity of astrocyte cultures was confirmed by labeling against glial fibrillary acidic protein (GFAP) and found to be greater than 95 %.

Electrophysiology

Whole-cell patch clamp recordings were obtained from neurons with an Axopatch-1C amplifier and pClamp software (Axon Instruments, Foster City, CA). External control solution contained (in mM): 140 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, 10 Glucose, and 6 Sucrose (pH 7.35). In Mg²⁺-free solution, Mg²⁺ was substituted by Ca²⁺ and 10 μ M glycine was added. Patch pipette solution contained (in mM): 140 K-gluconate, 10 EGTA, 4 Mg-ATP, 0.2 Tris-GTP, and 10 HEPES (pH 7.35). Unless stated otherwise, the membrane potential was held between -70 and -60 mV (in some cases while studying

IPSCs, the holding potential was -40 mV), where glutamatergic EPSCs and GABAergic IPSCs appeared as inward and outward currents, respectively (Basarsky et al., 1994; Trudeau et al., 1996).

The morphological identification of neurons was confirmed electrophysiologically by their ability to generate TTX-sensitive Na^+ -mediated action potentials and by the presence of fast synaptic currents. Confluent astrocytes, ~25-150 μm from the soma of the neuron recorded from in patch clamp recordings, were stimulated electrically or mechanically using glass micropipettes filled with external saline. For each neuron up to four different astrocytes were stimulated at intervals of greater than 1 minute using 4 voltage pulses (1 ms duration, 150 V) delivered at 10 Hz over the cellular surface. Similar results were obtained by mechanical stimulation elicited by gently tapping the astrocytes with a micropipette (Nedergaard, 1994). Unless stated otherwise, at least eight astrocytes were stimulated in each parallel control and test condition and data were obtained from at least three different cultures (i.e., at least 24 astrocytes stimulated in each condition). In some experiments, cells were incubated for 30-60 min with 1 μM thapsigargin.

Presynaptic neurons were extracellularly stimulated with glass micropipettes filled with external solution and placed over the soma. Suprathreshold voltage pulses (0.5 to 1 ms duration, 20 to 150 V) were continuously delivered at 0.5-1 Hz. Reductions in evoked synaptic currents were determined to be significant for each astrocyte-neuron pair by comparing the mean amplitude of synaptic currents evoked 30 s before and after astrocyte stimulation (Student's t-test comparison). Astrocyte-induced changes in spontaneous postsynaptic current (sPSC) frequency were determined for each cell pair by a similar method by comparing the mean frequency 15-30 s before and after astrocyte stimulation. The incidence of astrocyte-induced responses was defined as the proportion of responses relative to the total number of astrocytes on each experimental day. Therefore, for this variable, n values correspond to number of experiments, whereas for the other variables, such as the amplitude of the SIC, n represents the number of cells examined. Statistical differences were established using the Student's t-test unless stated otherwise. All

experiments were performed at room temperature (20-23 °C). Data are expressed as mean \pm SEM.

Tetanus toxin treatment

In some experiments we treated cultures with tetanus toxin to cleave the neuronal protein synaptobrevin and to block synaptic transmission. Cells were incubated overnight with 6.6 - 33 nM of tetanus toxin (holoprotein; Alomone Labs, Jerusalem, Israel) or carrier solution (control) in parallel cultures.

Immunocytochemistry

Neurons were identified by labeling with fluorescein-conjugated C-fragment of tetanus toxin (C-FITC, List Biological Laboratories, Inc., Campbell, CA) by a modification of a previously described procedure (Charles, 1994). Cells were incubated for 1 hour in 10 mg/ml of C-FITC at 37 °C. Following washing, cells were either viewed live or in other experiments were fixed with 4% paraformaldehyde in PBS at room temperature for 30 minutes for parallel immunocytochemistry. A monoclonal antibody (clone 69.1, 1:500; provided by Dr. R. Jahn) was used to probe for synaptobrevin II. Visualization was accomplished using rhodamine-conjugated secondary antibody and conventional epifluorescence microscopy.

Calcium measurements

The ability of electrical stimuli to evoke a wave of elevated calcium in astrocytes was monitored by fluorescence microscopy using fluo-3 as a calcium indicator. Cultures were loaded with fluo-3 by incubation at 37 °C for 45 minutes in the presence of fluo-3-AM (10 mg/ml; Molecular Probes, Eugene, OR). After washing, indicator was allowed to de-esterify for 45 minutes. Coverslips containing fluo-3 loaded cells were visualized using a silicon intensified target (SIT) camera (Hamamatsu) or IC-300 intensified CCD camera (Photon Technology International, Monmouth Junction, NJ) attached to a Nikon 300

inverted microscope and a NeD_{LC} optical workstation (Prairie Technologies, LLC; Waunakee, WI).

Microinjection of BAPTA into astrocytes

In some experiments we injected the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) into individual astrocytes to determine the requirement for a calcium elevation in astrocytes for astrocyte-neuron signaling. Microinjection pipettes with a tip diameter of ~400 nm were pulled from Kwik-Fil borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) using a Sutter P-2000 micropipette puller (Sutter Instrument Co., Novato, CA). Pipettes were filled with a solution containing 0.375 M BAPTA (pH 7.2) and 0.25 mM fluoro-ruby. This solution was pressure injected into single astrocytes by a 300 ms pulse of 15 p.s.i. using an Eppendorf micro-manipulator and a Narishige IM-200 micro-injector (Narishige, Greenvale, NY). Based on quantification of the fluoro-ruby fluorescence, we estimated that the injection led to about a 250-fold dilution of the pipette solution, resulting in a final intracellular BAPTA concentration of 1-2 mM.

RESULTS

Neuronal responses to astrocyte stimulation

To study astrocyte-neuron signaling we established mixed cultures of astrocytes and neurons isolated from postnatal hippocampus. A single astrocyte was electrically or mechanically stimulated (Fig. 1A) to cause an elevation of calcium in that cell. Subsequently, this calcium elevation spread to neighboring astrocytes in a propagating wave. To detect neuronal responses, we recorded in the whole cell configuration from adjacent neurons. Stimulation of astrocytes evoked three types of neuronal responses: *a*) a long-lasting, slow inward current (SIC), *b*) a long-lasting increase in the frequency of the sPSCs (excitatory and/or inhibitory; e.g., Fig. 1B2 and Fig. 7B), or *c*) both (Fig. 1B). This increase in postsynaptic currents is likely to be the result of astrocytes activating synapses which in turn release transmitter onto the neuron that is recorded from (see below).

The astrocyte-evoked neuronal responses were elicited by $75 \pm 2 \%$ (mean \pm SEM; $n = 26$ experiments) of stimulated astrocytes, with the SIC being detected in $46 \pm 3 \%$. The SIC developed slowly reaching a maximum amplitude of 37.5 ± 2.4 pA ($n = 235$; only currents ≥ 10 pA were considered astrocyte-evoked SIC, i.e., at least three times higher than the standard deviation of the baseline noise) after 1-15 seconds and lasted for a duration of up to 75 seconds (Fig. 1B1). The sPSC frequency increase was elicited by $18 \pm 2 \%$ of the stimulated astrocytes. The mechanism of the astrocyte-induced modulation of spontaneous synaptic transmission was not further investigated in this study. Astrocyte-induced neuronal responses were not prevented by 1 μ M tetrodotoxin (TTX; $73 \pm 8 \%$ and $75 \pm 6 \%$ in control and TTX, respectively; $n = 4$ experiments). In agreement with the presence of the SIC, stimulation of astrocytes elicited a slow, long-lasting neuronal depolarization that could trigger trains of action potentials (not shown) when recorded in current clamp mode as demonstrated previously by Hassinger et al. (1995).

Tetanus toxin C-FITC labeling of neuronal processes

In these experiments we applied stimuli from a pipette that is immediately above an astrocyte. Since phase-contrast optics were used to position the stimulating pipette, it is possible that a neuronal process as well as an astrocyte was stimulated. We therefore developed a methodology that would reveal the presence of neuronal processes in living preparations so that we could selectively stimulate neurite free regions of astrocytes. Previous studies have indicated that neurons, but not astrocytes, express receptors for tetanus toxin (Charles et al., 1994; Ahnert-Hilger and Bigalke, 1995). We confirmed the selective presence of tetanus toxin receptors in our cultures using the FITC-labeled C fragment of tetanus toxin. In mixed neuron and astrocyte cultures we found that tetanus toxin C-FITC labeled the soma and neurites of neurons (Fig. 2A). By contrast, when purified astrocyte cultures were used, tetanus toxin labeling was absent (Fig. 2B). Therefore, tetanus toxin C-FITC can be used to selectively label neurons in mixed cultures containing both astrocytes and neurons.

We made use of the ability to selectively label neurons to identify the location of neurites in mixed astrocyte-neuron cultures using tetanus C-FITC and phase-contrast microscopy. We guided our stimulating electrode to a region of an astrocyte which was devoid of neuronal processes (Fig. 2C, D), provided a local stimulus and determined the neuronal electrophysiological response. When the stimulation pipette was guided to a neurite free region of the astrocyte, specific stimulation of the astrocyte evoked a SIC in 75 % of the recorded neurons ($n = 12$). Since this labeling method might not reveal all neuronal processes we also used fluo-3, which we have demonstrated discloses all neurites (Parpura et al., 1994), to guide the stimulating electrode specifically to an astrocyte. Again, stimulation of the astrocyte reliably evoked neuronal SIC.

Since direct stimulation of the astrocyte leads to a neuronal response these data strengthen the notion that the neuronal responses shown in Fig. 1 represent responses to stimulation of the astrocyte rather than an effect of direct stimulation of the neuron. However, because of the concern that our labeling methods may not have disclosed all neuronal processes we determined the consequences of neuronal stimulation on postsynaptic recordings. We visually located neuronal processes and stimulated them extracellularly while recording the response in the soma, or alternatively, we stimulated presynaptic neurons. Direct stimulation of a process led to an action potential that was recorded in the soma as a fast inward current ($n = 14$), while presynaptic stimulation evoked synaptic currents (see Fig. 7-10). These short latencies events were not detected when we applied the stimulus to the astrocyte, further supporting the argument that we can selectively stimulate an astrocyte which gives rise to the neuronal responses shown in Fig. 1.

Astrocyte calcium elevation is necessary for astrocyte-induced neuronal SIC

To further control for non-specific effects of astrocyte stimulation we determined whether neuronal responses required the presence of a calcium wave in the astrocyte network. Charles et al. (1993) have previously demonstrated that calcium wave propagation between astrocytes is IP_3 -mediated and requires functional internal calcium

stores. By imaging fluo-3-loaded astrocytes we confirmed their earlier observation that depletion of internal stores by treatment with the Ca^{2+} -ATPase inhibitor, thapsigargin (1 μM), blocks the spread of the calcium wave between astrocytes (Fig. 3 A-D). We then incubated cultures in 1 μM thapsigargin while making electrophysiological recordings of neuronal currents. After thapsigargin treatment, the ability of astrocyte stimulation to evoke the SIC was significantly reduced (Fig. 3E; $n = 6$; $P < 0.001$). When the SIC was detected in thapsigargin, its mean amplitude (36.9 ± 4.8 pA) was not significantly different from that recorded in control (28.9 ± 3.4 pA). Since the incidence but not the amplitude of the SIC was affected by thapsigargin and because similar reductions were observed in the incidence of Ca^{2+} waves, it is likely that the remaining responses in thapsigargin were due to incomplete effects of the treatment. Taken together, these results are consistent with the hypothesis that the SIC is a result of the calcium wave in the astrocyte network.

Since thapsigargin could affect neuronal calcium homeostasis as well as astrocyte calcium waves, we developed a method that would selectively prevent calcium elevations in astrocytes. We microinjected the calcium chelator BAPTA into single astrocytes, together with a fluorescent indicator, fluoro-ruby, to label the injected cell. Since fluoro-ruby contains a dextran moiety (10,000 MW) conjugated to tetramethylrhodamine it does not pass through gap junctions and is maintained within the injected cell. First, we determined whether microinjection *per se* and whether injection of BAPTA affected calcium signaling in response to mechanical stimuli. Using cultures of purified astrocytes loaded with the calcium indicator fluo-3 we found that mechanical stimulation of uninjected cells increased calcium in the majority of cells that were directly stimulated (82 ± 8 %, $n = 9$ coverslips; Fig. 4C). To evaluate the ability of this stimulus to induce a calcium wave amongst adjacent astrocytes, we determined the proportion of non-stimulated cells within the field of view which responded with a calcium elevation (zero represents no wave, 100 % represents a wave amongst all cells in the field of view). On average 67 % of cells within one field of view participated in a calcium elevation ($n = 19$ trials; Fig. 4D). In parallel to these experiments on uninjected cells, single astrocytes were

microinjected with fluoro-ruby. Microinjection of fluoro-ruby did not significantly affect the ability of a mechanical stimulus to induce a calcium elevation in the directly stimulated cell (86 %), nor the propagation of a calcium wave (Fig. 4A, C, D).

We next microinjected BAPTA and fluoro-ruby into astrocytes and determined the ability of BAPTA (final concentration ~1 mM) to impair calcium waves. Stimulation of BAPTA injected cells only led to a calcium elevation in 28 % of the directly stimulated cells (Fig. 4B, C). Additionally, injection of BAPTA/fluoro-ruby into the stimulated cell significantly attenuated the calcium wave amongst astrocytes since on average only 12 % of adjacent cells responded with a calcium elevation as compared to 84 % in fluoro-ruby injected astrocytes (Fig. 4B, D; $p < 0.001$). Thus, microinjection of BAPTA specifically into one astrocyte significantly reduces the ability to generate calcium responses.

We made use of BAPTA injection into astrocytes to ask whether a calcium elevation within this non-neuronal cell is required for the generation of the neuronal SIC. Individual astrocytes were injected with BAPTA/fluoro-ruby or with fluoro-ruby alone. A fluoro-ruby positive cell was brought into the field of view and an adjacent neuron was recorded in the whole-cell configuration. Stimulation of BAPTA/fluoro-ruby injected astrocytes only produced a neuronal response in one out of seven experiments (14 %, $n = 7$; Fig. 4F, G). By contrast, stimulation of uninjected astrocytes, or stimulation of a fluoro-ruby injected astrocytes evoked neuronal SIC in 61 and 63 % of recorded cells respectively (Fig. 4E, G). Taken together these experiments demonstrate that selective stimulation of astrocytes leads to a calcium-dependent SIC in adjacent neurons.

The SIC is mediated by both NMDA and non-NMDA glutamate receptors

Using Ca^{2+} imaging techniques, two different mechanisms have been proposed to mediate astrocyte-neuron signaling. In mixed forebrain cultures from embryonic rats, Nedergaard (1994) found that this signal was attenuated by gap junction blockers, suggesting that it was mediated through intercellular connections rather than through a chemical transmitter. However, Parpura et al. (1994) reported that astrocyte-neuron signaling in postnatal cultures of visual cortices was mediated by glutamate released from

astrocytes acting on NMDA receptors, while Hassinger et al. (1995) found that both NMDA and non-NMDA glutamate receptors were responsible for this signal.

To distinguish between a gap junction and a glutamate-mediated generation of the neuronal SIC, we studied its pharmacology. The non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μ M) reduced the incidence of the SIC ($P < 0.01$), and the combination of CNQX and D-2-amino-5-phosphonopentanoic acid (D-AP5; 50 μ M; an NMDA glutamate receptor antagonist) abolished the SIC ($P < 0.001$) (Fig. 5A). Removal of external Mg^{2+} and the addition of 10 μ M glycine (Mg^{2+} -free solution) enhanced the magnitude of the SIC (Fig. 5B and C) without changing the proportion of astrocytes that evoked the SIC. D-AP5 significantly ($P < 0.001$) attenuated the magnitude of the SIC in Mg^{2+} -free solution (to 49 ± 11 pA, $n = 20$, from 318 ± 60 pA, $n = 19$; Fig. 5C). Neither the mGluR antagonist (S)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4; 0.5 mM) nor (S)- α -methyl-4-carboxyphenylglycine (MCPG; 0.5 mM) altered the incidence or amplitude of the astrocyte evoked SIC (Fig. 5A). Taken together, these results indicate that SIC was mediated by both NMDA and non-NMDA ionotropic, but not by metabotropic glutamate receptors.

The astrocyte-induced glutamate-dependent SIC is insensitive to tetanus toxin

While astrocytes were directly stimulated in our experiments, it is feasible that an astrocyte signal modulated glutamate release from neurons to produce the SIC. To test this possibility we incubated cultures in tetanus toxin, a highly selective protease that cleaves the synaptic protein, synaptobrevin, and renders neuronal synapses non-functional (Link et al., 1992; Schiavo et al., 1992). After treatment with tetanus toxin we then asked whether it blocked the astrocyte-induced neuronal SIC.

Because high concentrations of toxin receptors are present on nerve cells, but are practically absent on glial cells (Ahnert-Hilger and Bigalke, 1995; Fig. 2A, B), this toxin should act selectively on neurons. Although it has been reported that tetanus toxin can reduce glutamate release from astrocytes (Jeftinija et al., 1997), it is clear that short to intermediate periods of toxin incubation do not (Bezzi et al., 1998). We confirmed that

these receptors are preferentially expressed on neurons by incubating cultures in fluorescein-conjugated C-fragment of tetanus toxin (Fig. 2 and 6 A, B). To experimentally determine whether tetanus toxin acts selectively on neurons, we performed immunocytochemistry for the vesicle protein synaptobrevin, which is expressed in astrocytes as well as neurons, after incubation of cultures in the tetanus toxin holoprotein (Fig. 6 C, E and F) (Parpura et al., 1995b). Subsequent immunocytochemistry supported the selectivity of toxin action since it revealed an absence of synaptobrevin in neurons (Fig. 6D), but a retention of positive immunoreactivity in astrocytes (Fig. 6F).

As reported previously (e.g., Trudeau et al., 1996), incubation in tetanus toxin abolished both spontaneous and evoked synaptic transmission (Fig. 7A). Despite this abolition of synaptic transmission, stimulation of astrocytes still reliably evoked a SIC in neurons (Fig. 7B). Neither the proportion of astrocytes that evoked neuronal responses nor the mean SIC amplitude were significantly changed by bath application of tetanus toxin (Fig. 7C), indicating that the neuronal responses to astrocyte stimulation are due to glutamate released from the astrocytes. Since tetanus toxin abolished both inhibitory and excitatory synaptic currents, the astrocyte-induced increase in frequency of excitatory and inhibitory miniature synaptic currents (figure 2 and 7) is likely to be due to the activation of interposed synaptic terminals.

Astrocytes modulate both excitatory and inhibitory synaptic transmission

Since glutamate can modulate synaptic transmission (Forsythe and Clements, 1990; Baskys and Malenka, 1991; Zorumski et al., 1996), we explored the effects of stimulating astrocytes on synaptic transmission between cultured neurons. To monitor synaptic transmission we recorded whole-cell from one neuron while stimulating another with an extracellular patch pipette. Control experiments in which the extracellular pipette was placed adjacent to the recorded neuron, confirmed that each extracellular stimulus evoked a single action potential in the neuron. Additionally, when the pipette was placed over the neuron, stimuli that evoked action potentials did not simultaneously activate a calcium wave in the underlying astrocyte. After obtaining a baseline measurement of synaptic

transmission, an astrocyte was stimulated to induce a wave of elevated calcium. Astrocyte stimulation reduced the amplitude of both action potential-evoked excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs; Figures 8 and 9, respectively). The transient decrease in evoked synaptic transmission lasted for tens of seconds and was usually accompanied by an increase in the number of failures in synaptic transmission (Fig. 8B and 9B). The amplitude of evoked EPSCs and IPSCs was decreased by $33 \pm 4\%$ ($n = 18$) and $30 \pm 4\%$ ($n = 10$), respectively, and this reduction was induced by $62 \pm 9\%$ and $88 \pm 13\%$ of the stimulated astrocytes ($n = 4$ experiments). In control experiments in which astrocytes were not stimulated we confirmed that the magnitude of the synaptic current was essentially unchanged during the period of recording (99 % of initial amplitude; $n = 11$). While evoked synaptic currents were reduced in magnitude by astrocyte stimulation, the mean amplitude of miniature EPSCs (mEPSCs; $n = 17$) and IPSCs (mIPSCs; $n = 12$) (recorded in the presence of $1 \mu\text{M}$ TTX) were not significantly changed by astrocyte stimulation (Fig. 8C and 9C, respectively; Kolmogorov-Smirnov test).

To begin to address the mechanism of synaptic modulation by astrocytes we asked whether there was an obligatory relationship between the presence of the astrocyte-induced SIC and the reduction in evoked synaptic transmission. In 14 of 41 examples, astrocyte stimulation elicited both a SIC and the modulation of evoked synaptic transmission. In 14 other examples evoked synaptic transmission was modulated without an accompanying postsynaptic SIC while in six others a SIC was detected without an associated modulation of the synapse. Therefore, the astrocyte-induced SIC is not necessary for the modulation of synaptic transmission.

Presynaptic mGluRs have been shown to reduce both excitatory and inhibitory synaptic transmission in hippocampal neurons. A depression of inhibitory synaptic transmission is mediated by L-AP4-insensitive, MCPG-sensitive mGluRs (group I and group II in CA1 and CA3 pyramidal neurons, respectively; Gereau and Conn, 1995; Poncer et al., 1995; Fitzsimonds and Dichter, 1996). Excitatory synaptic transmission reduction is ubiquitously due to the activation of MAP4-sensitive group III mGluRs (Baskys and Malenka, 1991), although Group I (Gereau and Conn, 1995; Manzoni and Bockaert, 1995) or group II

mGluRs (Manzoni et al., 1995; Ugolini and Bordi, 1995; Vignes et al., 1995; Bushell et al., 1996; Yoshino et al., 1996) can also be involved depending on the hippocampal area, age and species considered. To determine whether the astrocyte-induced modulation of the synaptic transmission required the activation of mGluRs by glutamate released after astrocyte stimulation, we determined the sensitivity of this modulation to mGluR antagonists. The proportion of astrocytes that modulated EPSCs was significantly decreased by 0.5 mM MAP4 ($P < 0.05$) but unchanged by 0.5 mM MCPG (Fig. 10A), whereas the astrocyte-induced modulation of IPSCs was insensitive to MAP4 but antagonized by MCPG ($P < 0.01$) (Fig. 10B).

DISCUSSION

In this study we have used ion imaging and electrophysiological approaches to evaluate the functional consequences of astrocyte-neuron signaling on neuronal physiology. In order to study astrocyte-neuron signaling it was essential that selective stimuli were applied to the astrocyte while responses of the neuron were recorded. One experimental approach would have been to use neuromodulators which induce calcium elevations in astrocytes and then to have determined the consequences for the neuron. However, the concern that neuronal responses may be mediated at least in part by these modulators mitigated this approach. Instead we used mechanical and electrical stimuli that can be directed to localized regions of a cell. Following delivery of either stimulus to astrocytes, we determined that neurons responded with a glutamate-receptor dependent SIC and that this same stimulus could induce the modulation of action potential evoked synaptic transmission.

While it is clear that mechanical and electrical stimuli raise astrocyte calcium levels, care was taken to establish the selectivity of the stimulus. By staining neuronal processes with tetanus toxin C-FITC we were able to guide the stimulating pipette to areas of astrocytes in which neuronal processes were absent. Additionally, two experimental manipulations that prevent the stimulus-induced astrocyte calcium wave, thapsigargin and BAPTA injection, prevented the stimulus-induced neuronal responses. Of particular

importance was the microinjection of BAPTA. Since injection of this calcium chelator into one astrocyte prevented both the stimulus induced astrocyte calcium elevation as well as the neuronal SIC, we can conclude that the glutamate-dependent neuronal modulation is a consequence of the calcium elevation in the astrocyte.

Our present data extend the previous observations of astrocyte-neuron signaling (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995) by demonstrating that experimentally induced calcium elevations in astrocytes cause a delayed glutamate-dependent slow inward current in hippocampal neurons and a mGluR-dependent inhibition of inhibitory and excitatory synaptic transmission, which based on previous studies is likely to be presynaptic in origin (see e.g. Forsythe and Clements, 1990; Baskys and Malenka, 1991; Gereau and Conn, 1995). In support of a presynaptic modulation we detected an increase in the number of failures in evoked synaptic transmission with no associated change in amplitude of miniature synaptic currents. However, one might predict a simultaneous reduction in miniature frequency. In our experiments though miniature synaptic current frequency increased in some preparations. Since mGluR antagonists did not attenuate this increase in miniature frequency (not shown) we anticipate that it is due to the operation of a separate mechanism. Despite the sustained presence of glutamate during the SIC, desensitization of miniature synaptic currents was not detected. Since even low concentrations of extracellular glutamate can lead to receptor desensitization (Trussel and Fischbach, 1989; Zorumski et al., 1996), our data suggest that glutamate released from astrocytes does not act equally at all sites on the neuron. Perhaps there are discrete sites of release which spare receptors in the postsynaptic aspect of the synapse from desensitization but that permit activation of presynaptic mGluRs. Alternatively, glutamate uptake mechanisms may spare the synaptic cleft from a sustained elevation of glutamate during astrocyte-neuron signaling. Interestingly, type 2 mGluRs have been described to be localized at the preterminal zone rather than at the synaptic junction in mossy fiber-CA3 synapse (Yokoi et al., 1996), and such a spatial distribution of ionotropic and metabotropic receptors could account for the specific effects observed.

Astrocyte stimulation leads to three distinct neuronal consequences: a non-NMDA and NMDA-dependent ionotropic SIC, a mGluR-dependent presynaptic inhibition of transmitter release from synaptic terminals, and an increase in the frequency of "spontaneous" synaptic currents. Since astrocyte stimulation can depolarize neurons beyond their firing threshold, the astrocyte-induced increase in spontaneous PSC frequency might be easily explained by assuming that these PSCs are evoked by action-potentials. However, since TTX did not prevent that neuronal response, it is possible that the astrocyte directly influences the presynaptic terminal. The particular nature of this action, however, requires further investigation. Irrespective of mechanism, it is likely to be independent of mGluRs because agonists for these receptors either do not influence or reduce the frequency of TTX-insensitive miniature synaptic currents (Gereau and Conn, 1995).

There are at least three potential sources for glutamate to mediate the astrocyte-neuron signaling. First, astrocytes might stimulate the release of glutamate from neuronal synaptic terminals to induce the SIC. However, this is unlikely given that incubation of cultures in tetanus toxin cleaves the neuronal synaptic protein synaptobrevin and blocks neuronal exocytosis without impairing astrocyte-neuron signaling (Fig. 7). Additionally, our previous demonstration (Parpura et al., 1994) that elevated astrocyte calcium is both necessary and sufficient to induce the release of glutamate into the superfusate in purified astrocyte cultures indicates that glutamate can be provided by the astrocyte. Second, stimulation of the astrocyte might induce a depolarization-dependent reversal of glutamate transporters to elevate the extracellular levels of glutamate in the immediate environment of the neuron. Certainly, depolarization-induced transporter reversal can lead to glutamate actions on neurons when the astrocyte is recorded from with a patch pipette containing a reservoir of glutamate (Mennerick and Zorumski, 1994). However, glutamate transport inhibitors do not prevent stimulus-induced astrocyte-neuronal signaling (Parpura et al., 1994). This is perhaps not surprising given that glutamate is rapidly converted to glutamine in astrocytes by glutamine synthetase which would significantly reduce the concentration of this transmitter in the astrocyte cytosol. A third possibility is that a calcium-dependent exocytotic mechanism mediates glutamate release from astrocytes. While critical tests of

this hypothesis have not been performed the calcium dependence of astrocyte-neuronal signaling supports such hypothesis. Additionally, storage of glutamate in a vesicular compartment would overcome the problem of metabolism of cytosolic glutamate by glutamine synthetase activity.

Our studies point to a potential functional role for astrocytes in modulating neuronal activity and synaptic transmission *in vivo*. However, our study was performed in conditions far removed from the CNS since we used artificial stimuli to elevate astrocyte calcium levels within a cell culture environment. The question that results is whether such a signaling pathway could be activated *in vivo*. A recent study by Pasti et al. (1997) suggests that there can be bi-directional glutamate-dependent communication between astrocytes and neurons in acutely isolated hippocampal slices. These data suggest that astrocyte modulation of neuronal activity and synaptic transmission could well play a role in integration within the CNS. What are the signals that initiate calcium elevations in astrocytes and evoke astrocyte-induced neuromodulation? It has already been determined that glutamate released from neurons can lead to calcium elevations in adjacent astrocytes (Dani et al. 1992; Porter and McCarthy, 1996). Thus, "spillover" from glutamatergic synapses within the hippocampus might activate feedback modulation from astrocytes. Perhaps astrocytes contribute glutamate to the recently reported use-dependent activation of presynaptic metabotropic receptors (Scanziani et al., 1997). Another possibility is that the release of a neuronal transmitter, such as norepinephrine, a transmitter that has been demonstrated to elevate astrocyte calcium in acutely isolated slices (Duffy and MacVicar, 1995), would activate this astrocyte neuromodulatory pathway. While these possibilities need to be experimentally evaluated, we have previously demonstrated that a neuroligand, bradykinin, which mobilizes specifically astrocyte calcium, causes glutamate-dependent astrocyte-neuron signaling *in vitro* (Parpura et al., 1994). Given that this physiological stimulus is able to induce astrocyte-neuron signaling, coupled with the recent demonstration of bidirectional communication between astrocytes and neurons in hippocampal slice (Pasti et al., 1997), one must consider the possibility that astrocytes play an additional role in the CNS in modulating electrical activity and synaptic transmission.

ACKNOWLEDGMENTS

The authors would like to thank Drs. S. Shen, M. McCloskey, A. Charles and L.-E. Trudeau for their comments on drafts of the manuscript, Dr. R. Jahn for providing an antibody against synaptobrevin II and Lucia Pasti for providing a pre-print of her work. This work was supported by grants from the McKnight Foundation (P.G.H.) and the National Institutes of Health (P.G.H.), and by a NATO postdoctoral fellowship (A.A.) and a Long-term fellowship from the Human Frontier Science Program (A.A.).

ABBREVIATIONS

BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
C-FITC	fluorescein-conjugated C-fragment of tetanus toxin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
D-AP5	D-2-amino-5-phosphonopentanoic acid
EPSC	excitatory postsynaptic current
GFAP	glial fibrillary acidic protein
IPSC	inhibitory postsynaptic current
MAP4	(S)-2-amino-2-methyl-4-phosphonobutanoic acid
MCPG	(S)- α -methyl-4-carboxyphenylglycine
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current
mGluRs	metabotropic glutamate receptors
NMDA	N-methyl-D-aspartate
SIC	slow inward current
sPSCs	spontaneous postsynaptic currents
TTX	tetrodotoxin

REFERENCES

- Ahnert-Hilger, G. and Bigalke, H. (1995) Molecular aspects of tetanus and botulinum neurotoxin poisoning. *Prog. Neurobiol.*, **46**, 83-96.

- Basarsky, T.A., Parpura, V. and Haydon, P.G. (1994) Hippocampal synaptogenesis in cell culture: developmental time course of synapse formation, calcium influx, and synaptic protein distribution. *J. Neurosci.*, **14**, 6402-6411.
- Baskys, A. and Malenka, R.C. (1991) Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. *J. Physiol. (Lond.)*, **444**, 687-701.
- Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Lodi Rizzini, B., Pozzan, T. and Volterra, A. (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature*, **391**, 281-285.
- Bushell, T.J., Jane, D.E., Tse, H.W., Watkins, J.C., Garthwaite, J. and Collingridge, G.L. (1996) Pharmacological antagonism of the action of group II and III mGluR agonists in the lateral perforant path of rat hippocampal slices. *Br. J. Pharmacol.*, **117**, 1457-1462.
- Charles, A.C. (1994) Glia-neuron intercellular calcium signaling. *Dev. Neurosci.*, **16**, 196-206.
- Charles, A.C., Dirksen, E.R., Merrill, J.E. and Sanderson, M.J. (1993) Mechanisms of intercellular calcium signaling in glial cells studied with dantrolene and thapsigargin. *Glia*, **7**, 134-145.
- Charles, A.C., Merrill, J.E., Dirksen, E.R. and Sanderson, M.J. (1991) Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron*, **6**, 983-992.
- Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S. and Smith, S.J. (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signalling. *Science*, **247**, 470-473.
- Dani, J.W., Chernjavsky, A. and Smith, S.J. (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron*, **8**, 429-440.
- Duffy, S. and MacVicar, B.A. (1995) Adrenergic calcium signaling in astrocyte networks within the hippocampal slice. *J. Neurosci.*, **15**, 5535-5550.
- Finkbeiner, S.M. (1992) Calcium waves in astrocytes-filling in the gaps. *Neuron*, **8**, 1101-1108.

- Fitzsimonds, R.M. and Dichter, M.A. (1996) Heterologous modulation of inhibitory synaptic transmission by metabotropic glutamate receptors in cultured hippocampal neurons. *J. Neurophysiol.*, **75**, 885-893.
- Forsythe, I.D. and Clements, J.D. (1990) Presynaptic glutamate receptors depress excitatory monosynaptic transmission between mouse hippocampal neurons. *J. Physiol. (Lond.)*, **429**, 1-16.
- Gereau, R.W. and Conn, P.J. (1995) Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. *J. Neurosci.*, **15**, 6879-6889.
- Hassinger, T.D., Atkinson, P.B., Strecker, G.J., Whalen, L.R., Dudek, F.E., Koseel, A.H. and Kater, S.B. (1995) Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. *J. Neurobiol.*, **28**, 159-170.
- Jeftinija, S.D., Jefinija, K.V. and Stefanovic, G. (1997) Cultured astrocytes express proteins involved in vesicular glutamate release. *Brain Res.*, **750**, 41-47.
- Jensen, A.M. and Chiu, S.Y. (1990) Fluorescence measurement of changes in intracellular calcium induced by excitatory amino acids in cultured cortical astrocytes. *J. Neurosci.*, **10**, 1165-1175.
- Kriegler, S. and Chiu, S.Y. (1993) Calcium signalling of glial cells along mammalian axons. *J. Neurosci.*, **13**, 4229-4245.
- Link, E., Edelmann, L., Chou, J.H., Binz, T., Yamasaki, S., Eisel, U., Baumert, M., Sudhof, T.C., Niemann, H. and Jahn, R. (1992) Tetanus toxin action: inhibition of neurotransmitter release linked to synaptobrevin proteolysis. *Biochem. Biophys. Res. Commun.*, **189**, 1017-1023.
- Manzoni, O.J., Castillo, P.E. and Nicoll, R.A. (1995) Pharmacology of metabotropic glutamate receptors at the mossy fiber synapses of the guinea pig hippocampus. *Neuropharmacol.*, **34**, 965-971.
- Manzoni, O. and Bockaert, J. (1995) Metabotropic glutamate receptors inhibiting excitatory synapses in the CA1 area of rat hippocampus. *Eur. J. Neurosci.*, **7**, 2518-2523.

- Mennerick, S. and Zorumski, C.F. (1994) Glial contribution to excitatory neurotransmission in cultured hippocampal cells. *Nature*, **368**, 59-62.
- Murphy, T.H., Blatter, L.A., Wier, W.G. and Baraban, J.M. (1993) Rapid communication between neurons and astrocytes in primary cortical cultures. *J. Neurosci.*, **13**, 2672-2679.
- Nedergaard, M. (1994) Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science*, **263**, 1768-1771.
- Parpura, V., Basarsky, T.A., Liu, F., Jeftinija, K., Jeftinija, S. and Haydon, P.G. (1994) Glutamate-mediated astrocyte-neuron signalling. *Nature*, **369**, 744-747.
- Parpura, V., Fang, L., Brethorst, S., Jeftinija, K., Jeftinija, S., Haydon, P.G. (1995a) α -Latrotoxin stimulated glutamate release from cortical astrocytes in cell culture. *FEBS Lett.*, **360**, 266-270.
- Parpura, V., Fang, Y., Basarsky, T.A., Jahn, R. and Haydon, P.G. (1995b) Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP-25 in cultured astrocytes. *FEBS Lett.*, **377**, 489-492.
- Pasti, L., Volterra, A., Pozzan, T. and Carmignoto, G. (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes *in situ*. *J. Neurosci.*, **17**, 7817-7830.
- Poncer, J.C., Shinozaki, H. and Miles, R. (1995) Dual modulation of synaptic inhibition by distinct metabotropic glutamate receptors in the rat hippocampus. *J. Physiol. (Lond.)*, **485**, 121-134.
- Porter, J.T. and McCarthy, K.D. (1996) Hippocampal astrocytes *in situ* respond to glutamate released from synaptic terminals. *J. Neurosci.*, **16**, 5073-5081.
- Scanziani, M., Salin, P.A., Vogt, K.E., Malenka, R.C. and Nicoll, R.A. (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. *Nature*, **385**, 630-634.
- Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B. and Montecucco, C. (1992) Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*, **359**, 832-834.

- Trudeau, L.-E., Emery, D.G. and Haydon, P.G. (1996) Direct modulation of the secretory machinery underlies PKA-dependent synaptic facilitation in hippocampal neurons. *Neuron*, **17**, 789-797.
- Trussell, L.O. and Fischbach, G.D. (1989) Glutamate receptor desensitization and its role in synaptic transmission. *Neuron*, **3**, 209-218.
- Ugolini, A. and Bordi, F. (1995) Metabotropic glutamate group II receptors are responsible for the depression of synaptic transmission induced by ACPD in the dentate gyrus. *Eur. J. Pharmacol.*, **294**, 403-410.
- Verkhratsky, A. and Kettenmann, H. (1996) Calcium signalling in glial cells. *Trends Neurosci.*, **19**, 346-352.
- Vignes, M., Clarke, V.R., Davies, C.H., Chambers, A., Jane, D.E., Watkins, J.C. and Collingridge, G.L. (1995) Pharmacological evidence for an involvement of group II and group III mGluRs in the presynaptic regulation of excitatory synaptic responses in the CA1 region of rat hippocampal slices. *Neuropharmacol.*, **34**, 973-982.
- Yokoi, M., Kobayashi, K., Manabe, T., Takahashi, T., Sakaguchi, I., Katsuura, G., Shigemoto, R., Ohishi, H., Nomura, S., Nakamura, K., Nakao, K., Katsuki, M. and Nakanishi, S. (1996). Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. *Science*, **273**, 645-647.
- Yoshino, M., Sawada, S., Yamamoto, C. and Kamiya, H. (1996) A metabotropic glutamate receptor agonist DCG-IV suppresses synaptic transmission at mossy fiber pathway of the guinea pig hippocampus. *Neurosci. Lett.*, **207**, 70-72.
- Zorumski, C.F., Mennerick, S. and Que, J. (1996) Modulation of excitatory synaptic transmission by low concentrations of glutamate in cultured rat hippocampal neurons. *J. Physiol. (Lond.)*, **494**, 465-477.

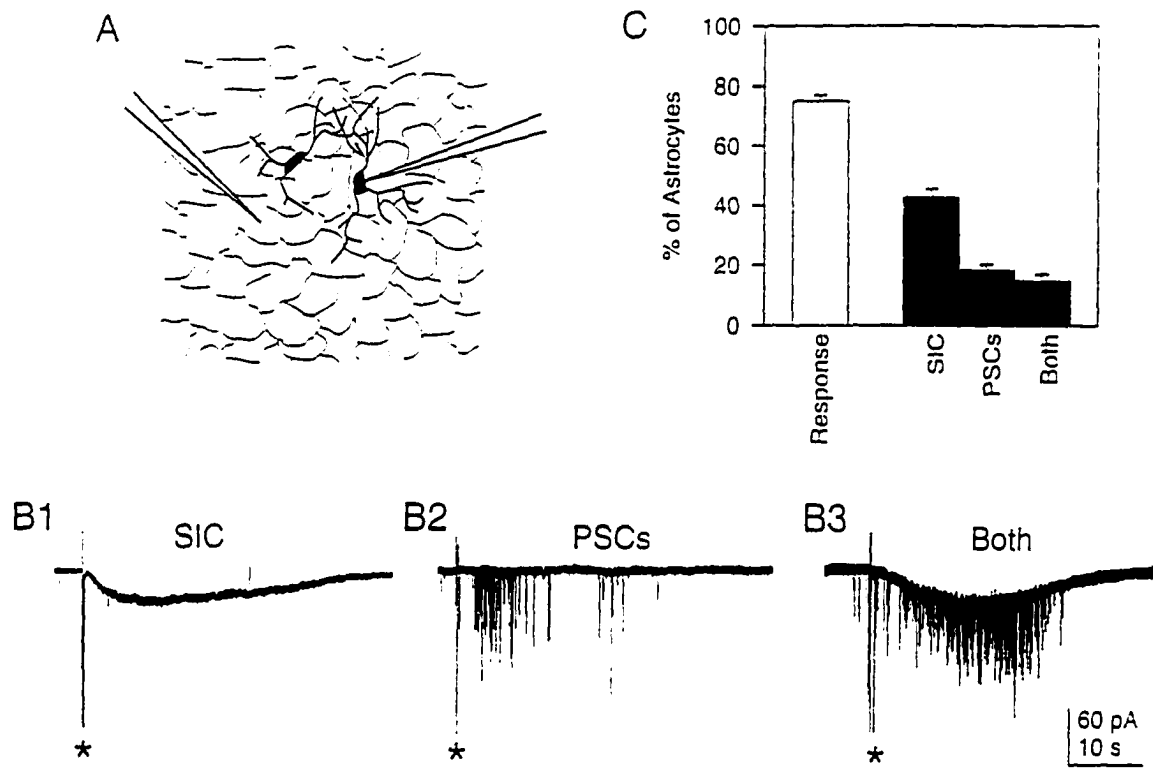


Figure 1

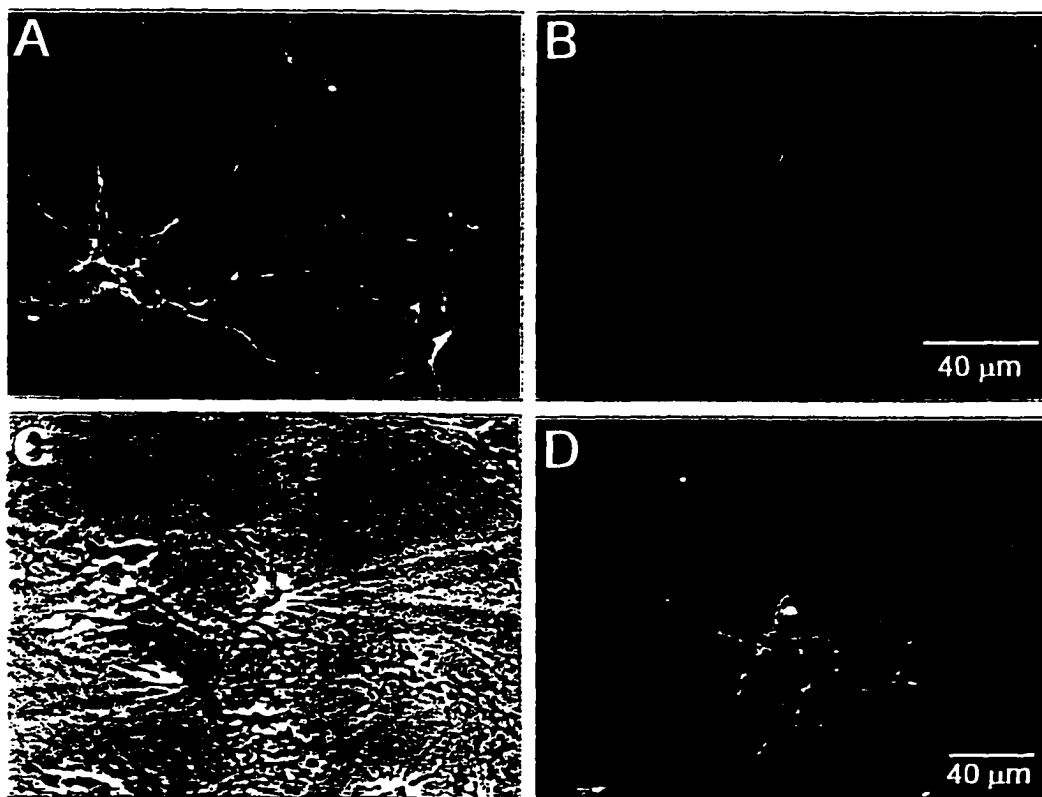


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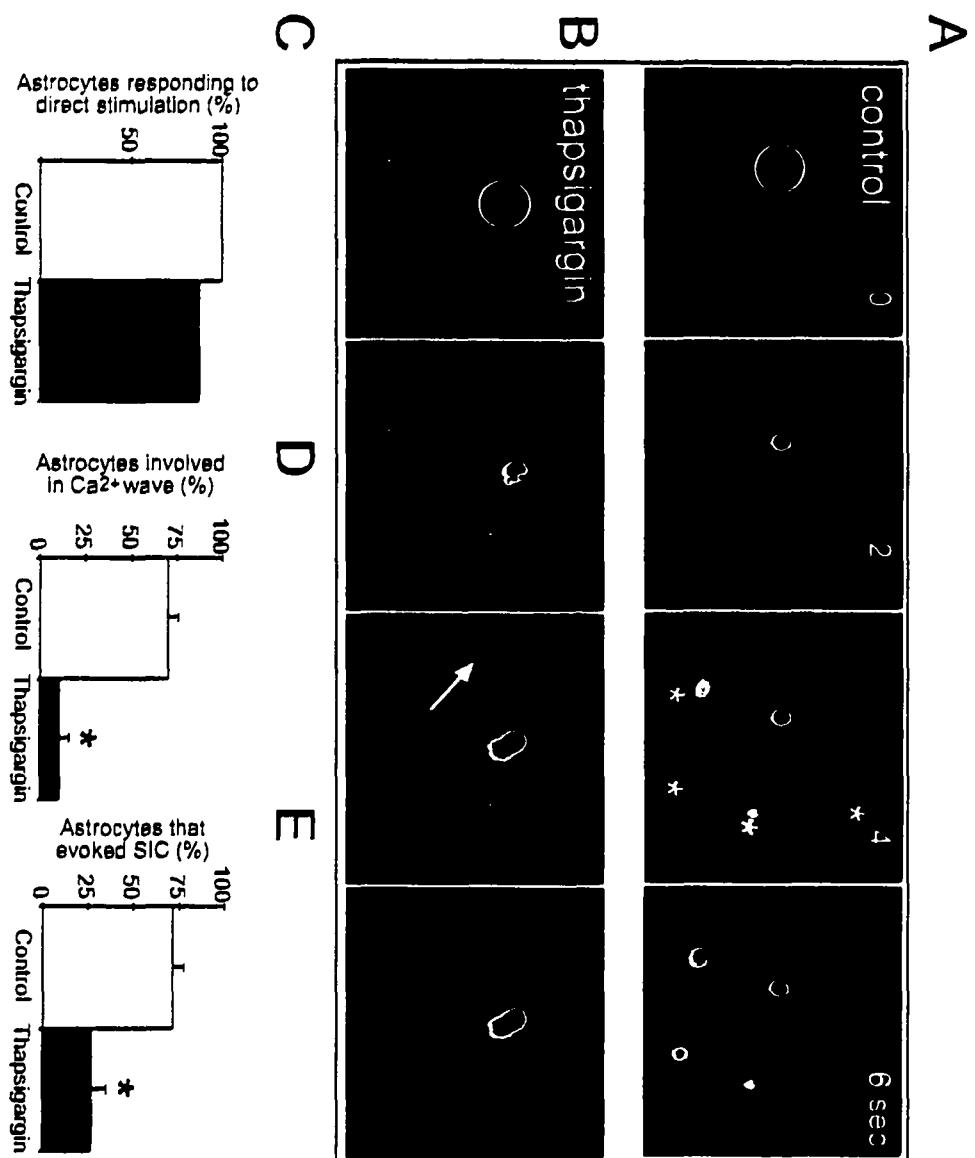


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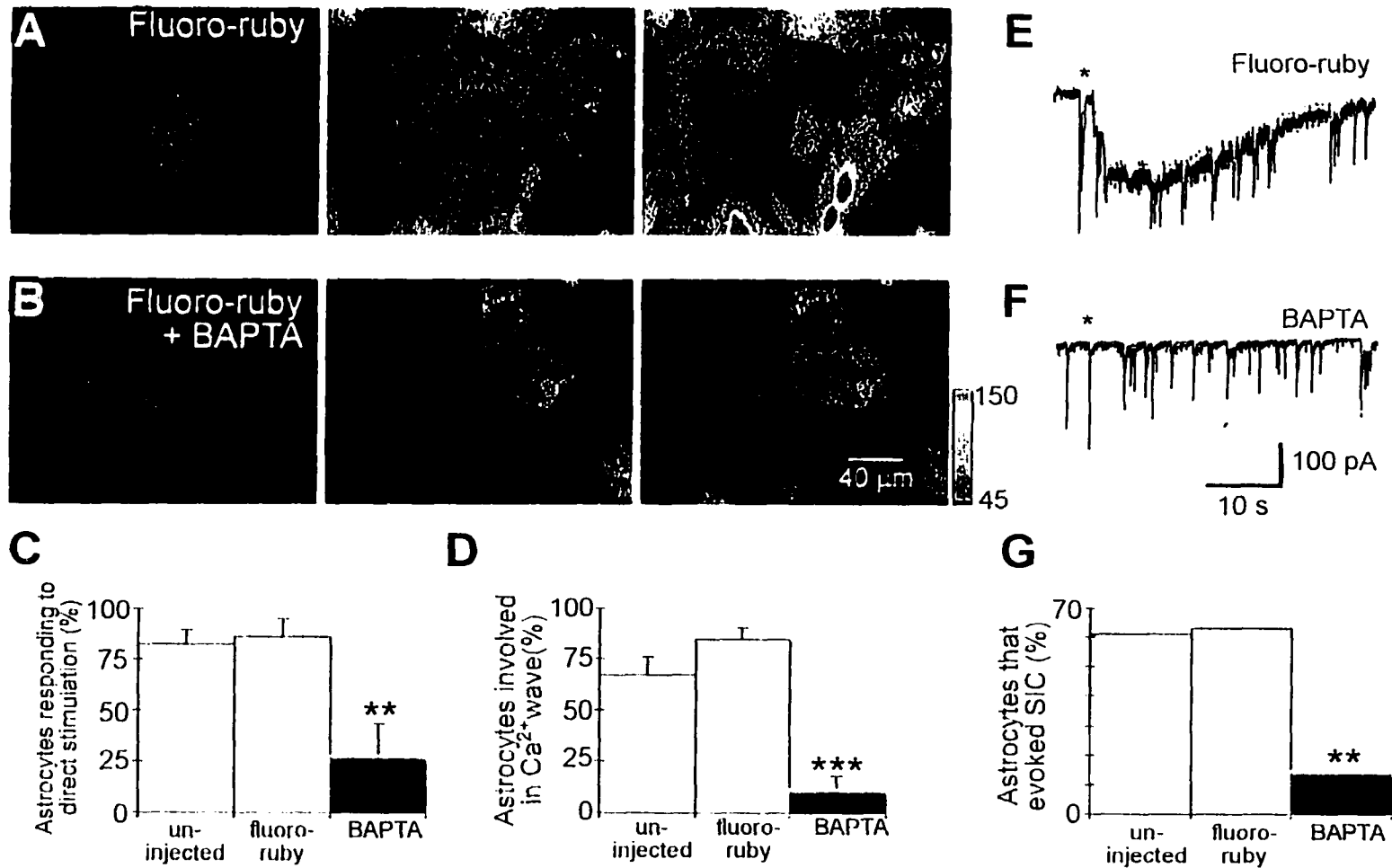


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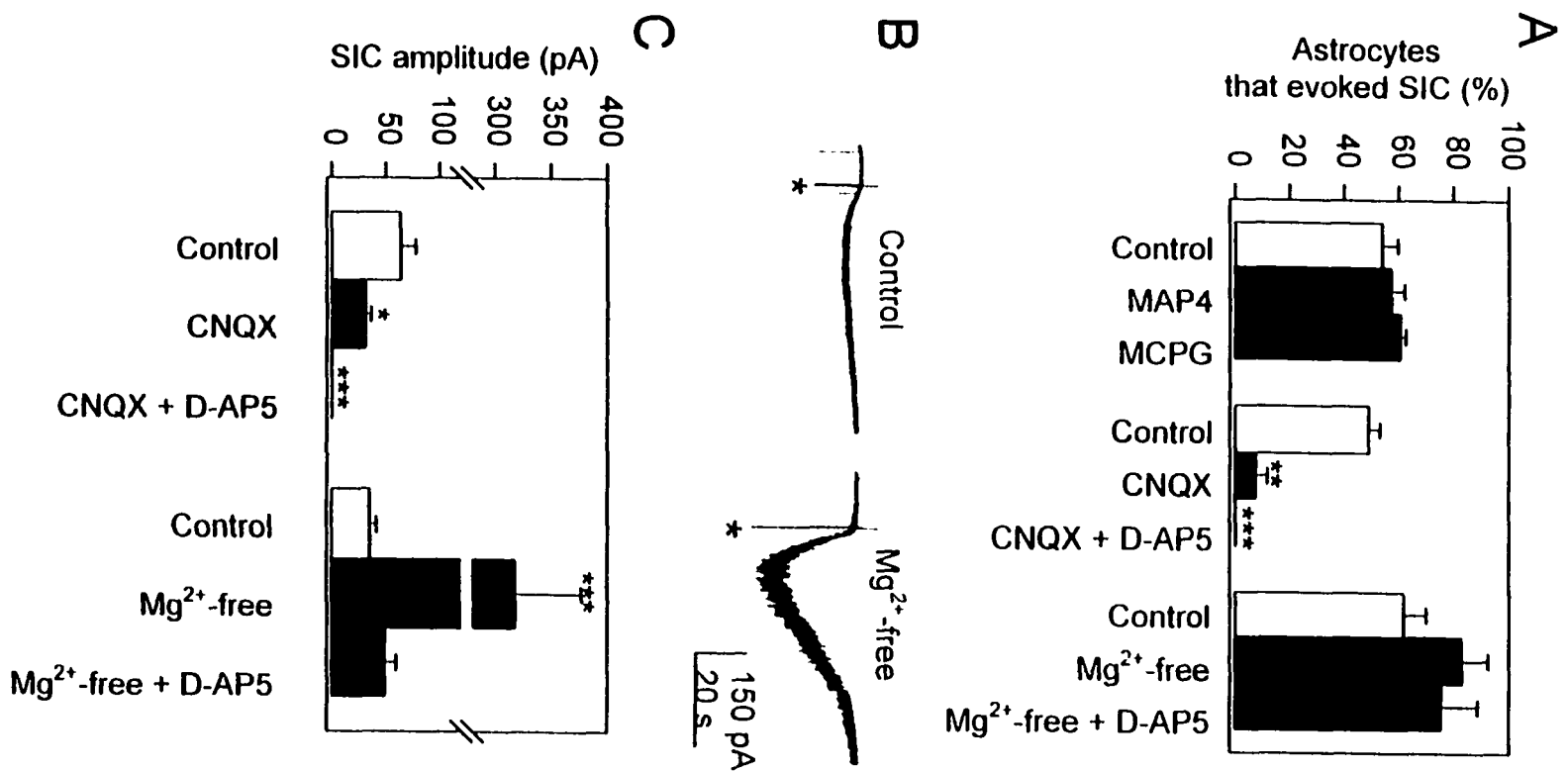


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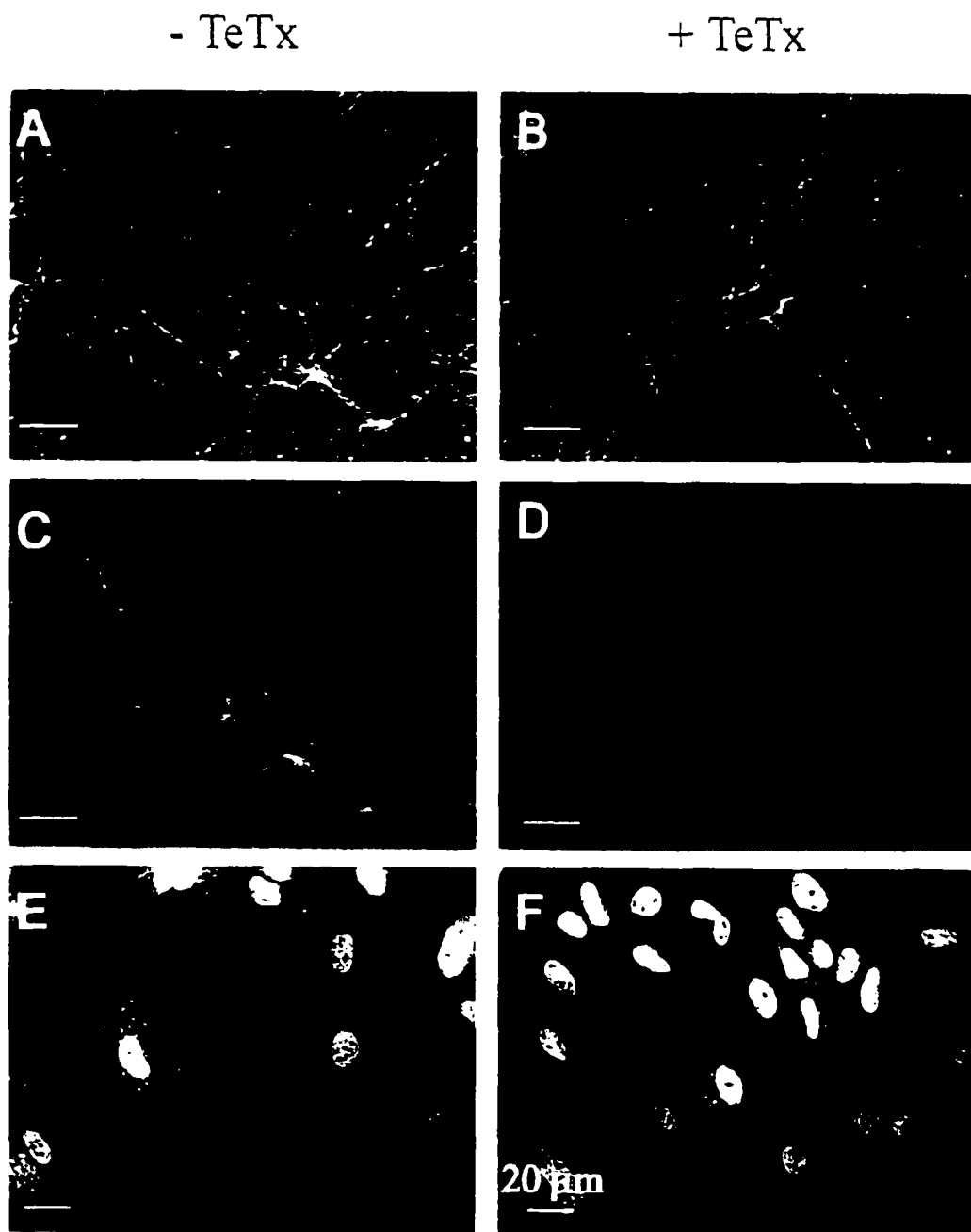


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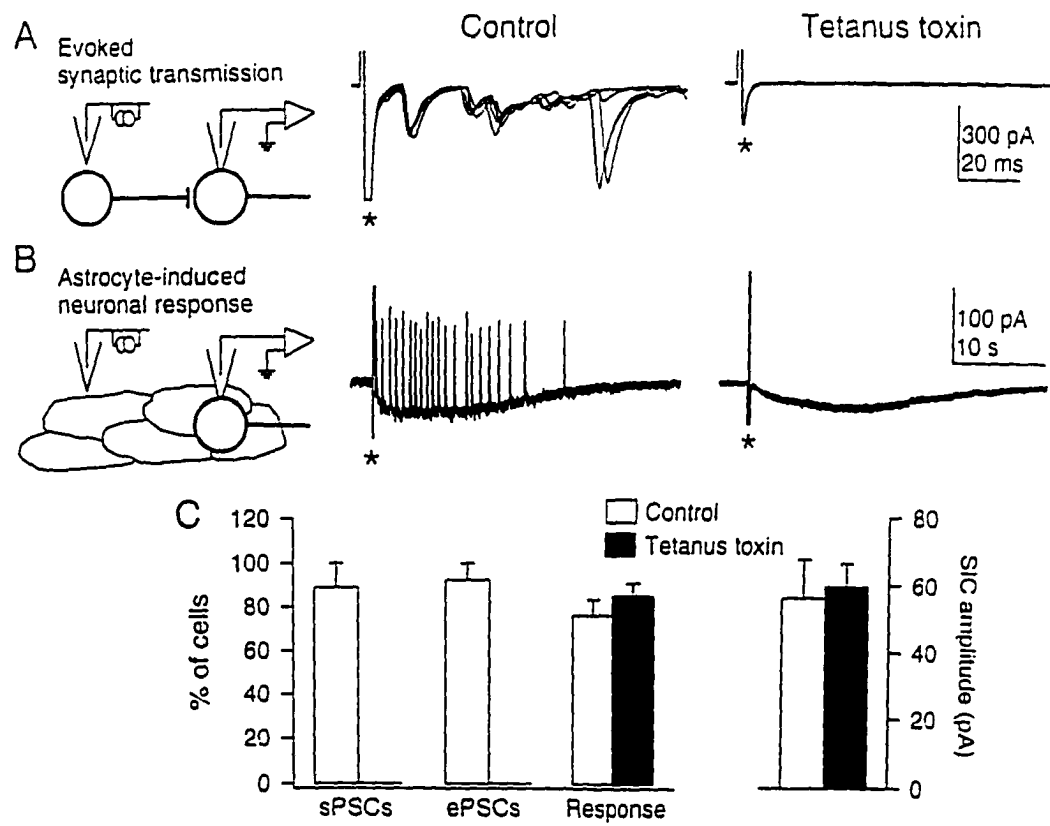


Figure 7

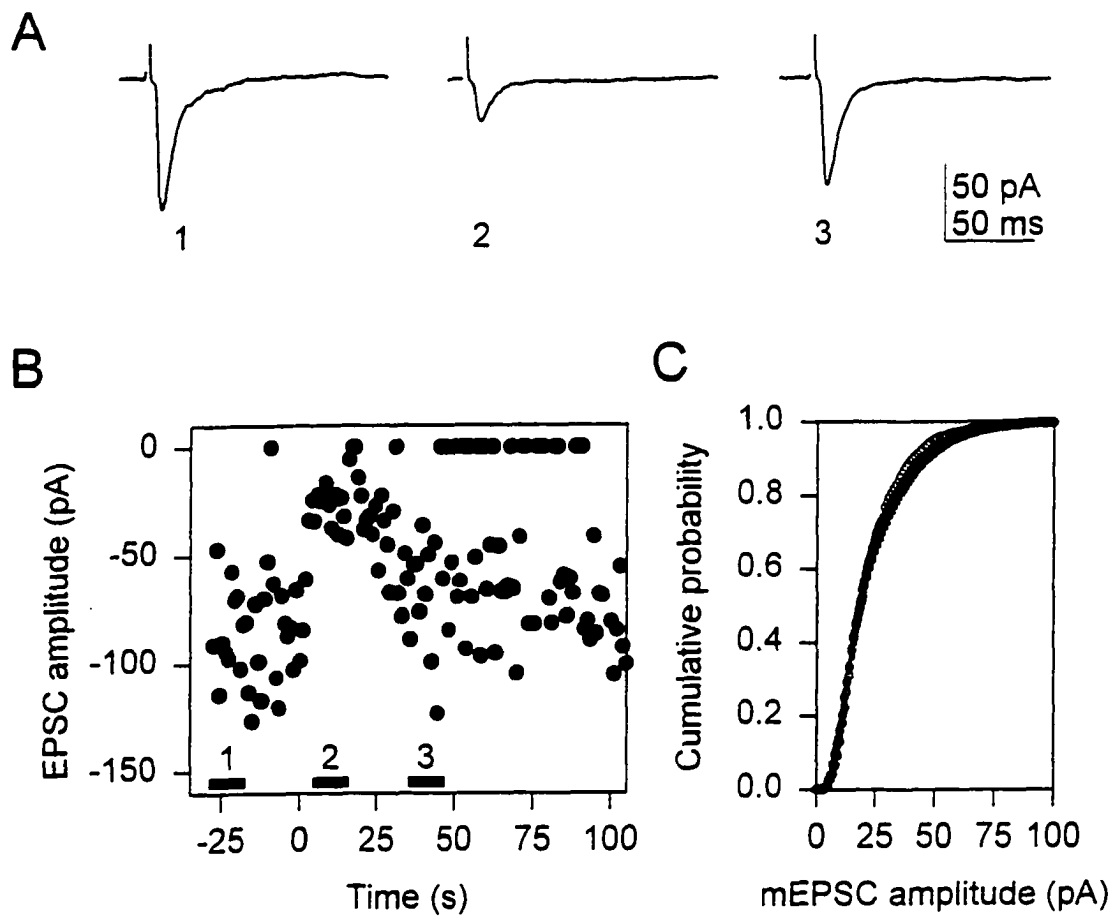


Figure 8

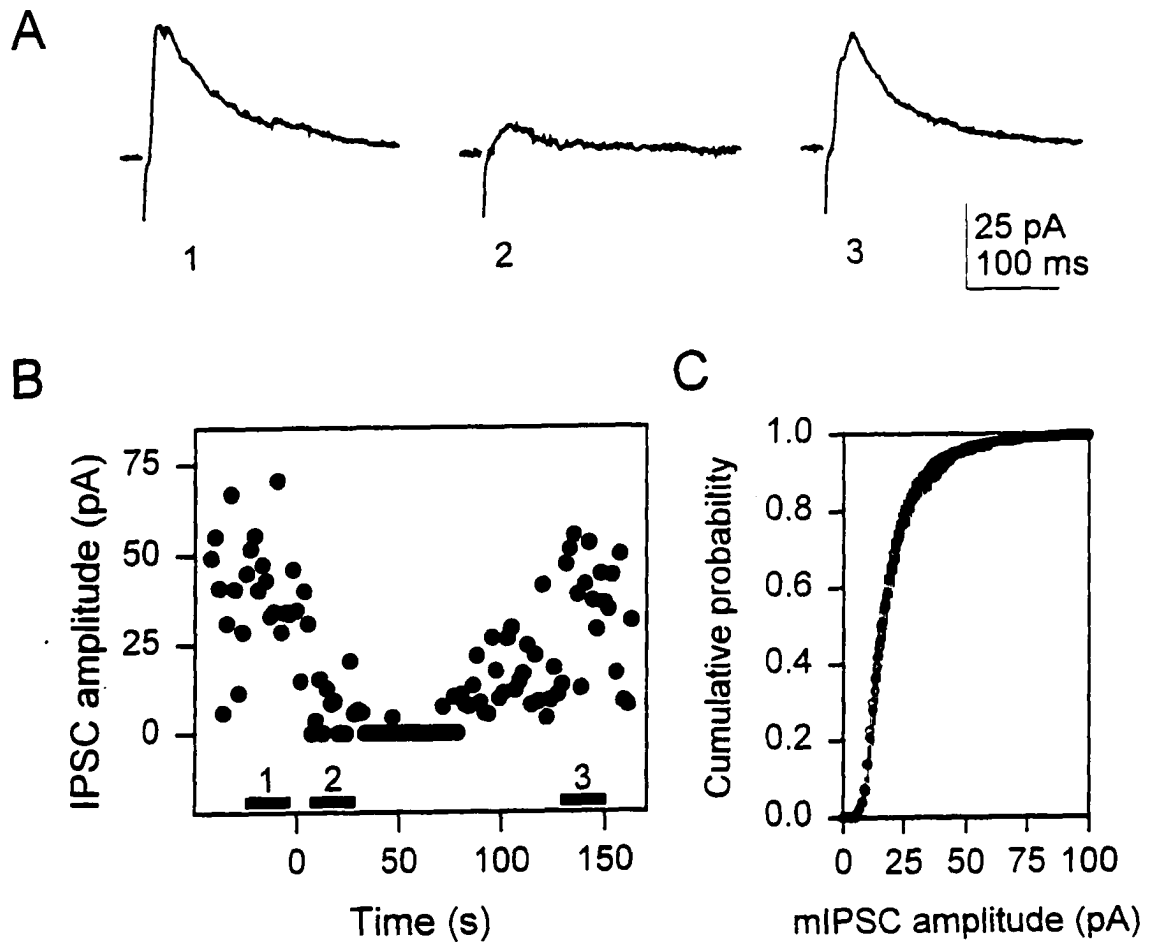
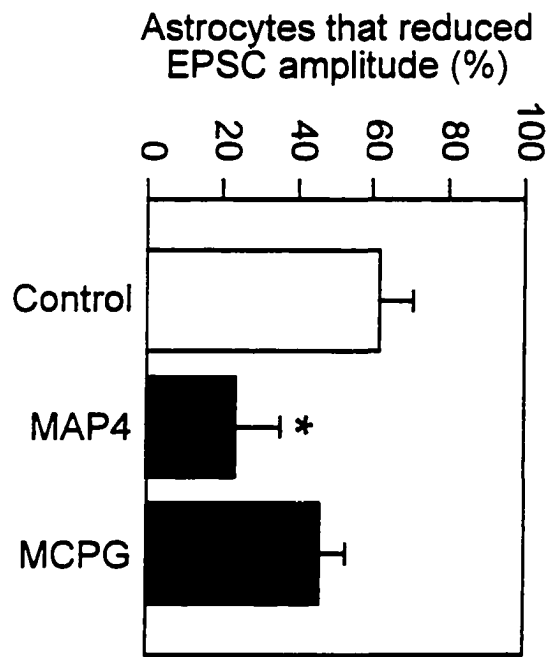


Figure 9

A



B

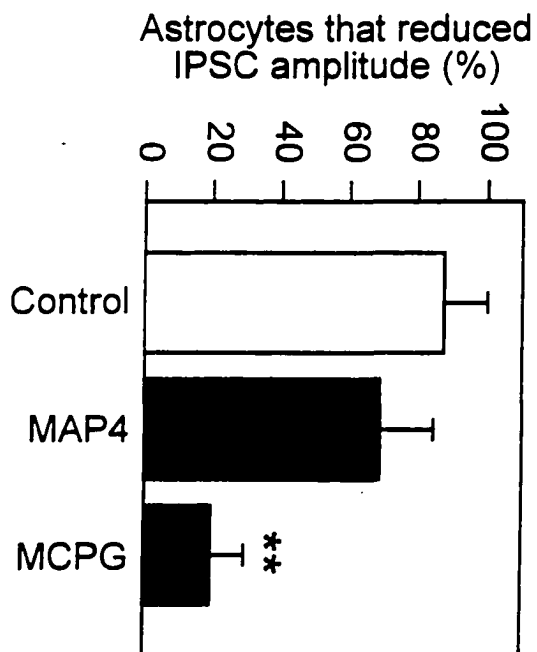


Figure 10

FIGURE LEGENDS

FIGURE 1. Neuronal responses to electrical stimulation of astrocytes. *A*, Schematic diagram representing a confluent layer of astrocytes (thin lines) and two neurons (thick lines). Astrocytes were mechanically or electrically stimulated with a glass micropipette, and whole-cell currents were recorded from adjacent neurons. To study the astrocyte-induced effects on synaptic transmission, the presynaptic neuron was electrically stimulated using a third electrode. *B*, Three typical neuronal responses were evoked by astrocyte stimulation: a long-lasting, slow inward current (SIC; *B1*), a long-lasting increase of spontaneous postsynaptic currents (PSCs; *B2*), or both (*B3*). Asterisks indicate stimulus artifacts. Note that these artifacts were only present when electrical stimuli were provided. *C*, Relative number of stimulated astrocytes that evoked neuronal responses and percentage of each type of astrocyte-evoked neuronal response ($n = 26$). Bars represent mean \pm SEM.

FIGURE 2. Selective labeling of neuronal processes with tetanus toxin C-FITC permits the stimulation of astrocytes in neurite-free regions. *A*, A mixed astrocyte neuron culture is labeled with the C fragment of tetanus toxin conjugated with FITC to identify tetanus toxin binding sites. The outline of neuronal somata and neuronal processes can be readily seen following labeling. To determine whether tetanus toxin C-FITC selectively labels neurons, the same reaction sequence as used in *A* was used on purified astrocyte cultures (*B*). Tetanus toxin C-FITC does not stain astrocytes. Living cultures were labeled with tetanus toxin C-FITC in order to reveal neurite free regions of astrocytes for stimulation while recording neuronal currents. The photomicrograph in *C* shows a phase-contrast image of a mixed culture in which astrocytes and neurons are readily visualized. To guide the astrocyte stimulating pipette shown in *C* tetanus toxin C-FITC was visualized with wide-field epi-fluorescence microscopy (*D*).

FIGURE 3. Thapsigargin prevents spreading of calcium elevation from stimulated astrocytes to adjacent cells and reduces the frequency of astrocyte-induced neuronal SIC. *A* shows a control experiment in which an astrocyte (*open circle*) was mechanically

stimulated with a glass micropipette. Calcium elevations occurred in the stimulated cell followed by calcium elevation in adjacent astrocytes (*asterisks*). In contrast, in *B*, treatment of a sister culture with 1 μ M thapsigargin prevents the spread of calcium elevation from the stimulated astrocyte (*open circle*) to adjacent cells (*arrow*). Mechanical stimuli were applied between the images that were acquired at time 0 and 2 seconds for both control and thapsigargin-treated astrocytes. Pseudocolor representation indicates intracellular calcium levels, where purple/blue represent basal calcium levels while yellow/red increased calcium levels. Quantitation of calcium imaging studies show that thapsigargin did not significantly reduce the ability of mechanical stimulation to induce a calcium elevation in the directly stimulated astrocyte (*C*), but significantly ($p < 0.001$) reduced the spread of the calcium wave to adjacent astrocytes (*D*). Cells were identified as participating in the Ca^{2+} wave if the fluo-3 signal changed $\geq 30\%$ of the baseline. In parallel electrophysiological experiments treatment of cultures with thapsigargin reduced the frequency with which astrocyte stimulation evoked a neuronal SIC (*E*; $p < 0.001$). These data suggest that a wave of calcium in astrocytes significantly contributes to astrocyte-neuron signaling. Numbers of repetitions are ≥ 8 in all categories. (* represents $p < 0.001$).

FIGURE 4. Microinjection of the calcium chelator BAPTA into an astrocyte prevents astrocyte calcium waves and blocks astrocyte-induced neuronal signaling. In *A* and *B*, purified astrocyte cultures were loaded with the calcium indicator fluo-3 to monitor the ability of mechanical stimulation to induce calcium elevations in astrocytes. In *A*, a control experiment is shown in which a single astrocyte was microinjected with a carrier solution containing fluoro-ruby (left panel). The center and right panels show two images in pseudocolor mode representing intensity of fluo-3 emission. The central panel is taken prior to mechanical stimulation of the fluoro-ruby injected cell. Mechanical stimulation increases intracellular calcium in the injected cell as well as in neighboring unstimulated astrocytes (right panel). In contrast, *B* shows the result of injecting fluoro-ruby and BAPTA into one astrocyte (left panel). Mechanical stimulation of the injected cell did not change the fluorescent emission of fluo-3 either in the stimulated cell or the neighboring

astrocytes (compare the central and right panels taken before and after mechanical stimulation). *C* and *D* show quantitative data taken from these experiments. BAPTA significantly reduces ($p < 0.01$) the ability of mechanical stimulation to elevate calcium levels in the directly stimulated cell (*C*) and the proportion of astrocytes involved in a stimulus-induced calcium wave ($p < 0.001$). In parallel studies neuronal currents were recorded in response to mechanical stimulation of astrocytes. While injection of fluoro-ruby did not prevent an astrocyte-induced SIC (*E, G*), injection of BAPTA prevented the appearance of a SIC following astrocyte stimulation (*F, G*; Chi-square test, $p < 0.01$). (** represents $p < 0.01$, *** represents $p < 0.001$). In all categories $n \geq 6$.

FIGURE 5. Astrocyte-evoked slow inward current is mediated by both NMDA and non-NMDA ionotropic glutamate receptors, but not by metabotropic glutamate receptors. *A*, Percentage of astrocytes that evoked a SIC in 0.5 mM MAP4, 0.5 mM MCPG, 20 μ M CNQX, 50 μ M D-AP5 and Mg^{2+} -free solution, and in their respective control solutions in parallel cultures. $n \geq 3$ for each group. *B*, SIC in control and Mg^{2+} -free solution, where the membrane current noise, probably NMDA receptor-mediated, conspicuously increased after astrocyte stimulation. Asterisks indicate stimulus artifacts. *C*, Mean amplitude of SIC detected under the different recording conditions. $n \geq 15$ for each group except for CNQX, where only four neurons had a detectable SIC due to the reduction in the number of responses. In CNQX + D-AP5, SIC amplitude was considered zero since no responses were observed. Significant differences with respect to control were established by the Student's *t*-test at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

FIGURE 6. Tetanus toxin cleaves synaptobrevin II in neurons, but not in astrocytes. *A*, Labeling of mixed astrocyte-neuron cultures with fluorescein-conjugated C-fragment of tetanus toxin specifically labels neurons in a punctate fashion. This punctate staining was apparent even after treatment with holoprotein tetanus toxin (TeTx) which cleaves synaptobrevin (*B*). The efficacy of tetanus toxin action on cells was determined by immuno-labeling of cultures with antibody against synaptobrevin II. Punctate pattern of

synaptobrevin II staining was present in untreated neurons (*C*; same culture field as shown in *A*), but not in tetanus toxin treated neurons (*D*; same field as in *B*). To determine whether the active form of tetanus toxin selectively cleaves synaptobrevin in neurons, purified astrocyte cultures were treated with the toxin. Anti-synaptobrevin II staining of astrocytes was unaffected by the tetanus toxin treatment (*E-F*). (Note that the synaptobrevin immunostaining of astrocytes is less intense than neurons making it necessary to both increase the excitation intensity and camera gain to detect positive immunoreactivity.) Scale bars indicate 20 μ m.

FIGURE 7. Tetanus toxin abolished spontaneous and evoked synaptic transmission but did not prevent astrocyte-evoked neuronal responses. *A*, EPSCs (5 superimposed traces) evoked by extracellular stimulation of a neuron in control conditions, were absent in tetanus toxin-treated cultures. The presence of EPSCs and IPSCs was assayed by extracellular stimulation of potential presynaptic neurons. Asterisks indicate stimulus artifacts. *B*, Astrocyte-evoked neuronal responses in control and tetanus toxin treated cells. Transient outward currents in control conditions correspond to astrocyte-evoked IPSCs. Note that after tetanus toxin treatment, astrocytes still evoked a neuronal SIC but neither spontaneous IPSCs nor EPSCs. *C*, Percentage of neurons with spontaneous and evoked postsynaptic currents (sPSCs and ePSCs, respectively; at least 16 postsynaptic and potential presynaptic neurons were tested in each group), and the percentage of astrocytes that evoked neuronal responses ($n \geq 34$ for each group), and amplitude of the SIC ($n \geq 21$ for each group) in control and tetanus toxin treated cells.

FIGURE 8. Astrocyte stimulation reduced the amplitude of evoked EPSCs. *A*, Averaged ($n = 10$) EPSCs evoked by extracellular stimulation at 1 Hz at the times indicated by the numbers in *B*. Holding potential was -60 mV. Holding currents are offset for illustration purposes. *B*, EPSC amplitude versus time. Zero time corresponds to the time of astrocyte stimulation. *C*, Cumulative probability distribution of the amplitude of mEPSCs (recorded in 1 μ M TTX) 30s before and after astrocyte stimulation (open and filled symbols,

respectively) ($n = 17$) shows that astrocyte stimulation does not change the amplitude of mEPSCs.

FIGURE 9. Astrocyte stimulation decreased the amplitude of evoked IPSCs. *A*, Averaged ($n = 10$) IPSCs evoked by extracellular stimulation at 0.5 Hz at the times indicated by the numbers in *B*. Holding potential was -60 mV. Currents are offset for illustration purposes. *B*, IPSC amplitude versus time. Zero time corresponds to the time of astrocyte stimulation. *C*, Cumulative probability distribution of the amplitude of mIPSCs (recorded in 1 μ M TTX) 30s before and after astrocyte stimulation (*open* and *filled symbols*, respectively) ($n = 12$). Holding potential was between -30 and -10 mV.

FIGURE 10. Astrocyte stimulation decreased evoked EPSCs and IPSCs through the activation of different mGluRs. *A*, and *B*, Proportion of astrocytes that when stimulated reduced the amplitude of evoked EPSCs and IPSCs, respectively, in control, 0.5 mM MAP4 and 0.5 mM MCPG. Significant differences with respect to control were established by the Student's t-test at $p < 0.05$ (*) and $p < 0.01$ (**).

CHAPTER 3. CALCIUM ELEVATION IN ASTROCYTES CAUSES AN NMDA RECEPTOR-DEPENDENT INCREASE IN THE FREQUENCY OF MINIATURE SYNAPTIC CURRENTS IN CULTURED HIPPOCAMPAL NEURONS

A paper submitted to the Journal of Neuroscience

Alfonso Araque, Rita P. Sanzgiri, Vladimir Parpura and Philip G. Haydon

My contribution is involved in testing the hypothesis that astrocyte Ca^{2+} elevation is necessary and sufficient for the astrocyte-induced increase in the mPSCs. As in Chapter 1, I micro-injected the Ca^{2+} chelator BAPTA into single astrocytes, along with the fluorescent dye fluoro-ruby to label the injected cell. I determined that the calcium chelator BAPTA, not micro-injection *per se*, prevented Ca^{2+} elevations in the injected cell as well as the intercellular Ca^{2+} waves in response to mechanical stimuli. I performed the micro-injections required for examining the effect of BAPTA on the neuronal SIC. To test the hypothesis that astrocyte Ca^{2+} elevation is sufficient to cause the neuronal responses, I injected the UV-sensitive Ca^{2+} cage NP-EGTA together with fluoro-ruby into specific astrocytes. These cells were used to confirm that photolysis increased intracellular Ca^{2+} and that this Ca^{2+} elevation further evoked neuronal responses. My work is included in the figures 3 and 4.

ABSTRACT

Astrocytes exhibit a form of excitability and communication based on intracellular Ca^{2+} variations (Cornell-Bell et al., 1990; Charles et al., 1991) which can be initiated by neuronal activity (Dani et al., 1992; Porter and McCarthy, 1996). A Ca^{2+} elevation in astrocytes induces the release of glutamate (Parpura et al., 1994; Pasti et al., 1997; Araque et al., 1998; Bezzi et al., 1998), which evokes a slow inward current in neurons, and

modulates action potential-evoked synaptic transmission between cultured hippocampal cells (Araque et al., 1998), suggesting that astrocytes and neurons may function as a network with bi-directional communication. Here we show that a Ca^{2+} elevation in astrocytes increases the frequency of excitatory as well as inhibitory miniature postsynaptic currents (mPSCs), without modifying their amplitudes. Thapsigargin incubation, microinjection of the Ca^{2+} chelator BAPTA, and photolysis of the Ca^{2+} -cage NP-EGTA demonstrate that a Ca^{2+} elevation in astrocytes is both necessary and sufficient to modulate spontaneous transmitter release. This Ca^{2+} -dependent release of glutamate from astrocytes enhances mPSC frequency by acting on N-methyl-D-aspartate (NMDA) glutamate receptors since it is antagonized by D-2-amino-5-phosphonopentanoic acid (AP5) or extracellular Mg^{2+} . These NMDA receptors are not located postsynaptically because blockage specifically of postsynaptic NMDA receptors by synaptic activation in the presence of the open channel blocker MK-801 did not impair the AP5-sensitive astrocyte-induced increase of mPSC frequency. Therefore, astrocytes modulate spontaneous excitatory and inhibitory synaptic transmission by increasing the probability of transmitter release through the activation of NMDA receptors that are likely present on the presynaptic terminal.

Key Words: astrocyte-neuron signaling; NMDA glutamate receptors; calcium cage photolysis;

calcium waves; miniature synaptic currents; hippocampal synaptic transmission.

INTRODUCTION

There is growing evidence indicating that astrocytes may play more active roles in the central nervous system beyond the simple structural and trophic support for neurons. The existence of signaling between astrocytes, using intracellular Ca^{2+} variations that can propagate as intercellular Ca^{2+} waves (Cornell-Bell et al., 1990; Charles et al., 1991; Finkbeiner, 1992; Newman and Zahs, 1997), the susceptibility of astrocytes to respond to neuronal activity (Dani et al. 1992; Porter and MacCarthy, 1996) by activation of neurotransmitter receptors that trigger Ca^{2+} waves (Cornell-Bell et al., 1990; Charles et al.,

1991; Murphy et al., 1993; Duffy and MacVicar, 1995), and the ability of astrocytes to signal to neurons by releasing glutamate that induces a neuronal Ca^{2+} elevation (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Pasti et al., 1997; Araque et al., 1998; Bezzi et al., 1998) suggest that astrocytes and neurons may function as a network where bi-directional communication takes place.

Although the existence of communication between astrocytes and neurons is firmly established by Ca^{2+} imaging studies (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Pasti et al., 1997), the physiological consequences of such communication have not been elucidated. However, we have recently shown that electrical or mechanical stimulation of astrocytes evoke in adjacent neurons a glutamate-dependent slow inward current mediated by activation of both N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors (Araque et al., 1998). We have also provided evidence for the involvement of astrocytes in the modulation of action potential-evoked synaptic transmission, through the activation of presynaptic metabotropic glutamate receptors (mGluRs). In addition to these electrophysiological consequences of the astrocyte-neuron signal, we have also reported that astrocyte stimulation may increase the frequency of miniature postsynaptic currents (mPSCs) through undefined mechanisms. Therefore, the aim of the present work was to characterize the mechanisms underlying this astrocyte-induced modulation of mPSC frequency.

We used three different stimuli, mechanical, electrical, or UV photolysis of a Ca^{2+} -cage, to raise the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in astrocytes, while mPSCs were recorded in adjacent neurons. We found that astrocyte stimulation transiently increased the frequency of excitatory as well as inhibitory mPSCs, without modifying their amplitude distribution. The elevation of intracellular Ca^{2+} in astrocytes was both necessary and sufficient to induce an increase in mPSC frequency, and was prevented by the NMDA receptor antagonist D-2-amino-5-phosphonopentanoic acid (AP5), indicating that it was mediated by activation of NMDA receptors. Since the astrocyte-induced increase in mPSC frequency was not impaired after the selective block of postsynaptic NMDA receptors with the use-dependent open-channel blocker MK-801, we conclude that astrocytes can increase

the frequency of mPSCs by activating non-synaptic NMDA receptors that are potentially located on the presynaptic terminal.

MATERIALS AND METHODS

Culture preparation

Primary cultures of mixed hippocampal neurons and astrocytes from 1- to 3-day-old postnatal rats were prepared as previously described (Araque et al., 1998) and were used after 8-25 days in culture, at a time when synapses were established. At the time of use, astrocytes were confluent in these cultures.

Electrophysiology

Whole-cell patch clamp recordings were obtained from neurons with an Axopatch-1C amplifier and pClamp software (Axon Instruments, Foster City, CA). Currents were filtered at 1-2 kHz and sampled above 2 kHz. External control solution contained (in mM): 140 NaCl, 5 KCl, 4 CaCl₂, 10 HEPES, 10 Glucose, and 6 Sucrose (pH 7.35). mPSCs were recorded in 1 μ M tetrodotoxin (TTX). To optimize NMDA receptor activation, Mg²⁺ was omitted and 10 μ M glycine was added to the solution. High osmolarity solution was obtained by adding 0.3 M sucrose. In 4 mM Mg²⁺ solution, MgCl₂ was added without osmolarity compensation. The patch pipette solution contained (in mM): 140 K-gluconate, 10 EGTA, 4 Mg-ATP, 0.2 Tris-GTP, and 10 HEPES (pH 7.35). In some experiments, 10 mM BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; tetrapotassium salt) was added to the pipette solution. Unless stated otherwise, the membrane potential was held at -60 mV to study miniature excitatory postsynaptic currents (mEPSCs), at -10 mV to analyze miniature inhibitory postsynaptic currents (mIPSCs), or at -30 mV to permit simultaneous observation of both mEPSCs and mIPSCs. At the holding potentials used, glutamatergic EPSCs and GABAergic IPSCs were identified as inward and outward currents, respectively (Araque et al., 1998). For thapsigargin treatment, cells were incubated with 1 μ M thapsigargin for 30-60 min. In some experiments, synaptic transmitter release was increased by using high osmolarity solution (0.3 M sucrose was added to the

control solution) delivered from a micropipette (tip diameter $\sim 2 \mu\text{m}$) by 1-10 s duration pressure pulses (Picospritzer II, General Valve, Fairfield, NJ).

The morphological identification of neurons was confirmed electrophysiologically by their ability to generate TTX-sensitive Na^+ -mediated action potentials and by the presence of fast synaptic currents. Confluent astrocytes $\sim 25\text{-}150 \mu\text{m}$ from the soma of the recorded neuron, were stimulated mechanically using glass micropipettes filled with external saline (see Nedergaard, 1994; Charles, 1994; Araque et al., 1998). Similar results were obtained when voltage pulses (1 ms duration, 150 V) were used to stimulate astrocytes (see Araque et al., 1998). However, to prevent potential effects of direct electrical stimulation on synaptic terminals, results presented were largely obtained using mechanical stimulation, unless stated otherwise. At least eight astrocytes were stimulated in each parallel control and test condition and data were obtained from at least three different experiments (i.e., at least 24 astrocytes stimulated in each condition). The incidence of astrocyte-induced responses was defined as the proportion of responses relative to the total number of astrocytes stimulated in each experiment, and statistical differences were established using the Student's t-test. All experiments were performed at room temperature (20-23 °C). Data are expressed as mean \pm SEM.

Analysis of mPSCs was done using the ACSPLouF software (obtained from Dr. Pierre Vincent, University of California at San Diego). The cumulative probabilities of the mPSC amplitude and frequency before and after astrocyte stimulation were plotted. The mPSC frequency was calculated in 1 s bins. Cumulative probability plots 15-30 s before and after astrocyte stimulation were compared, and significant differences were established at $p < 0.05$ using the Kolmogorov-Smirnov test.

Neuronal labeling with tetanus toxin

Neurons were identified by labeling with fluorescein-conjugated C-fragment of tetanus toxin (C-FITC, List Biological Laboratories, Inc., Campbell, CA) by a modification of a previously described procedure (Charles, 1994) as reported elsewhere (Araque et al.,

1998). Cells were incubated for 1 hour in 10 $\mu\text{g/ml}$ of C-FITC at 37°C. Following a wash period, cells were viewed using fluorescein optics.

Calcium measurements

The ability of stimuli to evoke a wave of elevated Ca^{2+} in astrocytes was monitored by fluorescence microscopy using the Ca^{2+} indicator fluo-3. Cultures were incubated at 37 °C for 45 minutes with the acetoxymethyl ester of fluo-3 (fluo3-AM, 10 mg/ml; Molecular Probes, Eugene, OR). After washing, the indicator was allowed to de-esterify for 45 minutes. Coverslips containing fluo-3 loaded cells were visualized using a silicon intensified target (SIT) camera (Hamamatsu Photonic System, Bridgewater, NJ) or IC-300 intensified CCD camera (Photon Technology International, Monmouth Junction, NJ) attached to a Nikon 300 inverted microscope and a NeD_{LC} optical workstation (Prairie Technologies, LLC; Waunakee, WI). Quantitative fluorescence measurements were made using the NeD_{LC} video software.

Microinjection into astrocytes

In some experiments we injected the Ca^{2+} chelator BAPTA into individual astrocytes as described elsewhere (Araque et al., 1998). Microinjection pipettes (tip diameter of ~ 400 nm) were pulled from Kwik-Fil borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) using a Sutter P-2000 micropipette puller (Sutter Instrument Co., Novato, CA). Pipettes were filled with a solution containing 375 mM BAPTA (pH 7.2) and 0.25 mM fluoro-ruby. This solution was pressure injected into single astrocytes using a 300 ms. 15 p.s.i pulse (Narishige IM-200, Narishige, Greenvale, NY). Positioning of micropipettes was controlled by an Eppendorf micro-manipulator. Based on quantification of the fluoro-ruby fluorescence, the resulting final intracellular BAPTA concentration was estimated to be 1-2 mM (Araque et al., 1998). In other experiments, NP-EGTA (o-nitrophenyl EGTA, tetrapotassium salt; Molecular Probes, Eugene, OR) was injected into individual astrocytes from pipettes filled with 15 mM NP-EGTA and 0.25 mM fluoro-ruby.

RESULTS

We have previously demonstrated that stimuli that elevate $[Ca^{2+}]_i$ in astrocytes can induce an increase in the frequency of mPSCs in adjacent neurons (Araque et al., 1998). To further evaluate the mechanism responsible for this response, we mechanically stimulated astrocytes while miniature synaptic currents were recorded from adjacent neurons. The frequency of both mEPSCs and mIPSCs was increased by mechanical stimulation of astrocytes (Fig. 1 and 2). The mEPSCs frequency was increased following stimulation of $48.6 \pm 3.8\%$ of astrocytes ($n = 20$). In those cells which responded to astrocyte stimulation, mEPSC frequency increased reaching a maximum (mean maximum increase was 12.2 ± 2.6 times the control frequency; $n = 21$) and declined slowly, usually lasting for about 1-2 minutes before returning to pre-stimulus values (Fig. 1B). This increase in frequency of miniature synaptic currents was also obvious by comparing the cumulative probability plots of the mEPSC frequency 30 s before and after astrocyte stimulation (Fig. 1C, open and filled symbols, respectively; $p < 0.005$). This increased mEPSC frequency was not accompanied by perceptible changes in the profile of the histograms of the mEPSC amplitudes (Fig. 1D and E) nor by statistically significant changes in the corresponding cumulative probability plots (Fig. 1F and G). Likewise, mIPSC frequency increase was elicited by $37.2 \pm 3.7\%$ of stimulated astrocytes ($n = 26$), the mean maximum increase in mIPSC frequency was 11.7 ± 2.3 times the prestimulus level ($n = 22$), and it showed a similar time course to the change in mEPSC frequency (Fig. 2A-C). Moreover, astrocytes increased the frequency of mIPSCs without modifying their amplitude (Fig. 2D-G). Taken together, these data suggest that stimulation of the astrocyte caused an increase in the probability of presynaptic transmitter release from both excitatory and inhibitory neurons.

Elevation of Ca^{2+} in astrocytes is both necessary and sufficient to modulate synaptic transmission

To control for the possibility that our mechanical stimuli directly activate neuronal processes, we used the FITC-labeled C fragment of tetanus toxin, that selectively labels the soma and neurites of the neurons but not astrocytes, thus allowing visualization of neuronal

processes in living preparations and permitting the selective stimulation of neurite-free regions of astrocytes (Araque et al., 1998). Using this method to disclose neuronal processes, specific stimulation of astrocytes still evoked an increase in mPSC frequency (13 out of 24 astrocytes). Although we cannot discount the possibility that unlabelled processes were still present in these cultures, these results suggest that astrocytes are responsible for increasing the mPSC frequency.

We have previously demonstrated that an elevation of Ca^{2+} in astrocytes is necessary to evoke astrocyte-induced slow inward current in adjacent neurons (Araque et al., 1998), therefore, we asked whether a Ca^{2+} elevation in these cells was also necessary for the increase in mPSC frequency. Astrocyte Ca^{2+} waves require functional internal Ca^{2+} stores, that can be depleted with the Ca^{2+} -ATPase inhibitor, thapsigargin (Charles et al., 1993; Newman and Zahs, 1997; Araque et al., 1998). After thapsigargin incubation, mechanical stimulation of astrocytes no longer led to a Ca^{2+} wave (not shown, but see Araque et al., 1998), and the ability of astrocyte stimulation to increase the frequency of mPSCs was significantly reduced (mEPSCs: 61.1 ± 3.5 % in control and 3.3 ± 3.3 % in thapsigargin; $p < 0.001$, mIPSCs: 47.9 ± 9.5 % in control and 8.9 ± 5.0 % in thapsigargin; $p < 0.01$), without altering their baseline frequency (the resting mean mEPSC frequency in control was 2.98 ± 1.86 s⁻¹, and 3.30 ± 1.24 s⁻¹ in thapsigargin-treated cells; $n = 8$). While we cannot exclude the possibility that thapsigargin acted through neuronal Ca^{2+} stores, these results suggest that a Ca^{2+} elevation in astrocytes is required for the stimulus-induced increase in mPSC frequency.

To further control for non-specific effects of stimulation, Ca^{2+} elevations were specifically blocked in astrocytes by microinjecting the Ca^{2+} chelator BAPTA into individual astrocytes. BAPTA was injected into a single astrocyte, along with the fluorescent indicator fluoro-ruby (a dextran-conjugated dye that does not pass through gap junctions, thus only labeling the injected cell) (Fig. 3A and B, left panels), and the astrocyte Ca^{2+} wave propagation was monitored using the Ca^{2+} indicator fluo-3. Stimulation of an uninjected astrocyte reliably evoked (18 out of 22) an increase in its intracellular Ca^{2+} that resulted in Ca^{2+} increments in adjacent astrocytes. Injection of fluoro-

ruby alone did not change the ability of astrocytes to respond to direct stimulation nor to evoke Ca^{2+} waves (Fig. 3A, C and D). However, the injection of BAPTA significantly reduced the proportion of stimulated astrocytes that responded with a Ca^{2+} elevation as well as reduced the proportion of cells involved in the Ca^{2+} wave (Fig. 3B-D). Similarly, injection of BAPTA into astrocytes, but not of fluoro-ruby alone, significantly reduced the ability of astrocyte stimulation to increase the frequency of mPSCs (Fig. 3E-G). Data obtained from experiments with C-FITC-tetanus toxin labeling, thapsigargin treatment, and BAPTA microinjection when taken together demonstrate that a Ca^{2+} elevation, specifically in astrocytes, is necessary for the stimulus-induced increase in mPSC frequency.

To further investigate the role of Ca^{2+} in the astrocyte-induced modulation of synaptic transmission, we asked whether a Ca^{2+} elevation in astrocytes was sufficient to increase the frequency of mPSCs. Single astrocytes were microinjected with the UV-sensitive Ca^{2+} -cage NP-EGTA (together with the fluorescent dye fluoro-ruby to identify the injected cell) that was photolyzed using one to six 4 ns UV (337 nm) pulses delivered through a UV transmitting optical fiber (beam diameter 10-15 μm) (Parpura and Haydon, in preparation). Photolysis reliably increased intracellular Ca^{2+} as monitored using fluo-3 (4 out of 4 cells). In parallel experiments analyzing the effects on neuronal currents, photolysis reliably evoked the previously characterized non-synaptic slow inward current (Araque et al., 1998) as well as an increase in the frequency of mPSCs (Fig. 4A and B). In contrast, UV stimulation of either uninjected astrocytes or fluoro-ruby injected astrocytes, did not change the mPSC frequency (Fig. 4B). Taken together these experiments demonstrate that an elevation of astrocyte Ca^{2+} is both necessary and sufficient to induce an increase in the frequency of both inhibitory and excitatory miniature synaptic currents.

Astrocyte-induced synaptic enhancement is mediated by activation of NMDA receptors

Several studies have demonstrated that an elevation of internal Ca^{2+} in astrocytes induces a Ca^{2+} -dependent release of glutamate that can be sensed by adjacent neurons (Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Pasti et al., 1997; Araque et al.,

1998; Bezzi et al., 1998). We used pharmacological tools to determine the glutamate receptor dependence of the astrocyte-induced increase in mPSC frequency (Fig. 5). The incidence of the astrocyte-mediated increase of mIPSC or mEPSC frequency was unaffected by the mGluR antagonists (S)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4; 0.5 mM) or (S)- α -methyl-4-carboxyphenylglycine (MCPG; 0.5 mM). The non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μ M; since mEPSCs are sensitive to this antagonist, the effect of CNQX on miniature frequency was assayed only on mIPSCs) did not reduce the ability of astrocytes to induce an increase in mIPSC frequency. However, either the presence of 4 mM Mg^{2+} or of the NMDA glutamate receptor antagonist AP5 (50 μ M) dramatically reduced the astrocyte-induced increase in mIPSC and mEPSC frequency. Additionally, in photolysis experiments, AP5 blocked the ability of photolytic Ca^{2+} elevations in astrocytes to raise mPSC frequency (see Fig. 4B). These data indicate that the enhancement of the miniature synaptic frequency was selectively mediated by the activation of NMDA receptors.

Astrocyte-induced mini frequency increase is not mediated by postsynaptic NMDA receptors

Current mechanistic models for the induction of some forms of synaptic plasticity postulate that NMDA receptor-dependent plasticity arises from the activation of postsynaptic NMDA receptors (see e.g., Manabe et al., 1992; Malgaroli and Tsien, 1992; Bliss and Collingridge, 1993; Wyllie et al., 1994; Nicoll and Malenka, 1995). To critically evaluate the contribution of postsynaptic NMDA receptors in the astrocyte-evoked synaptic modulation, we selectively blocked them with MK-801, an irreversible, use-dependent open channel blocker of NMDA receptors (Hessler et al., 1993; Rosenmund et al., 1993; Reid et al., 1997), and asked whether the AP5-sensitive astrocyte modulation of mPSC frequency persisted (Fig. 6A).

Previous studies have shown that EPSCs decay with bi-exponential kinetics that reflect two components: a fast CNQX- and a slow AP5-sensitive component (e.g., Bekkers and Stevens, 1989; McBain and Dingledine, 1992). We first confirmed in our experimental

control conditions that mEPSCs showed a fast ($\tau = 5.6 \pm 0.8$ ms) and a slow ($\tau = 45.1 \pm 11.1$ ms) component ($n = 6$) (Fig. 6B, control), and that the latter was abolished by 50 μ M AP5, whereas the fast component remained unchanged ($\tau = 6.6 \pm 0.8$ ms; not shown). To block postsynaptic NMDA receptors, we applied MK-801 (5 μ M) while activating synapses with high osmolarity solution (1-50 s duration pressure pulses of solution with 0.3 M sucrose added) (Fig. 6A). After this treatment, the decay phase of the mEPSCs could be fitted by a single exponential with a time constant (6.6 ± 1.6 ms) that was not significantly different from the fast time constant in control (Fig. 6B, MK-801), confirming that postsynaptic NMDA receptors were blocked. Additional evidence that MK-801 had blocked the postsynaptic NMDA receptors was provided by the inability of AP5 to further modify the mEPSC decay time constant (6.0 ± 0.6 ms and 6.4 ± 0.7 ms before and after AP5, respectively; $n = 4$).

Once the block of postsynaptic NMDA receptors was confirmed, we determined whether astrocytes could increase the frequency of mPSCs. In spite of the blockade of postsynaptic NMDA receptors by MK-801, stimulation of astrocytes still induced an increase in mPSC frequency that was sensitive to AP5 (Fig. 6A and C). Since postsynaptic NMDA receptors were blocked by MK-801, yet astrocytes still evoked an AP5-sensitive increase in mPSC frequency, we conclude that postsynaptic NMDA receptors do not mediate this form of synaptic modulation.

To further evaluate the role of the postsynaptic NMDA receptors in mediating the astrocyte-induced increase in mPSC frequency, we dialyzed the Ca^{2+} chelator BAPTA (10 mM) in the postsynaptic cell, and incubated cells with NO-Arg (50 μ M) that inhibits the production of nitric oxide, a candidate retrograde messenger putatively involved in some forms of synaptic plasticity (Schuman and Madison, 1991; Haley et al., 1992). Neither of these manipulations affected the ability of astrocytes to modulate the frequency of mPSCs (see Fig. 5), suggesting that changes in the postsynaptic neuron and specifically in its $[\text{Ca}^{2+}]_i$ are not necessary to trigger the astrocyte-induced synaptic modulation.

DISCUSSION

Recent experimental evidence presented by several laboratories has challenged the traditional idea of astrocytes as being supporting cells for neurons, and have suggested a more active role for astrocytes in the nervous system. It has been shown that astrocytes exhibit a form of excitability based on intracellular Ca^{2+} variations and that they can communicate between themselves through propagating intercellular Ca^{2+} waves (Cornell-Bell et al., 1990; Charles et al., 1991; Finkbeiner, 1992; Newman and Zahs, 1997). Moreover, astrocytes, that express several neurotransmitter receptors (Cornell-Bell et al., 1990; Charles et al., 1991; Murphy et al., 1993; Duffy and MacVicar, 1995), may respond to neuronal activity by generating Ca^{2+} waves (Dani et al., 1992; Porter and MacCarthy, 1996), indicating that neurons can signal to astrocytes. Furthermore, astrocytes may also signal to neurons because astrocyte stimulation can induce Ca^{2+} elevations in neurons (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Pasti et al., 1997; Araque et al., 1998; Bezzi et al., 1998). Therefore, these results suggest that neurons and astrocytes may function as an interdependent network with bi-directional communication between these elements.

However, in spite of the recent accumulating data describing the existence as well as mechanisms of astrocyte-neuron signaling, very little is known about their physiological implications. In a first report addressing that question, we determined the consequences of astrocyte stimulation on electrophysiological properties and on action potential-evoked synaptic transmission of hippocampal neurons (Araque et al., 1998). We demonstrated that astrocytes can evoke glutamate-dependent, NMDA and non-NMDA-mediated slow inward currents in neurons. We have also shown that astrocytes can modulate action potential-evoked synaptic transmission by activation of presynaptic mGluRs. In the present study, we present additional physiological consequences of astrocyte stimulation on neuronal function. Indeed, results presented above show that astrocyte stimulation may increase the frequency of both mEPSCs and mIPSCs. One concern, however, is whether our stimuli, which we direct to the astrocyte, do specifically elevate the Ca^{2+} level only in the astrocyte. Experiments using thapsigargin, intracellular BAPTA injection into single astrocytes, and

single-cell UV photolysis of NP-EGTA support previous findings indicating that intracellular Ca^{2+} elevation in astrocytes is both necessary and sufficient to evoke glutamate release and thus the glutamate receptor-dependent neuronal responses.

It is classically considered that changes in the frequency of mPSCs reflect a modification in the probability of the presynaptic transmitter release (see e.g., Del Castillo and Katz, 1954; Malgaroli and Tsien, 1992; Manabe et al., 1992; Wyllie et al., 1994). We have shown that astrocyte stimulation increased the frequency of mPSCs without modifying their amplitude distribution, suggesting that astrocytes induced an alteration of the presynaptic terminal, increasing the probability of transmitter release. Contrastingly, the astrocyte-induced increase in mPSC frequency is blocked by AP5, suggesting that it could be mediated by a postsynaptic NMDA receptor-dependent signaling, through the activation of a retrograde messenger (Schuman and Madison, 1991; Haley et al., 1992; Herrero et al., 1992) or other signaling pathway, as proposed for long-term potentiation in hippocampal CA3-CA1 synapses (see e.g., Malgaroli and Tsien, 1992; Manabe et al., 1992; Bliss and Collingridge, 1993; Nicoll and Malenka, 1995; Barria et al., 1997). However, since the blockade of postsynaptic NMDA receptors by synaptic activation in the presence of MK-801, postsynaptic dialysis of BAPTA, and addition of NO-arg did not attenuate the astrocyte-induced increase in mPSC frequency, it is unlikely that a postsynaptic NMDA receptor-dependent signaling pathway contributes to this action. While we cannot totally rule out the possibility that extrasynaptic NMDA receptors located on the postsynaptic cell mediate the astrocyte-induced synaptic effects, an alternative interpretation is that NMDA receptors located in the presynaptic terminal, but not in the synaptic cleft *per se*, mediate the effects of astrocyte stimulation.

The presence of NMDA receptors in presynaptic terminals from many areas of the CNS (including hippocampus) has been postulated by numerous studies using immunocytochemical localization (e.g., Aoki et al., 1994; Liu et al., 1994; Siegel et al., 1994; Johnson et al., 1996; Van Bockstaele and Colago, 1996) and biochemical analysis of neurotransmitter release (e.g., Fink et al., 1990; Pittaluga and Raiteri, 1992; Krebs et al., 1991; Martin et al., 1991; Desce et al., 1992; Cheramy et al., 1996; Wang and Thukral,

1996). Furthermore, it has been recently reported (Fu et al., 1995) that acetylcholine-mediated mPSCs can be potentiated by glutamate through the activation of presynaptic receptors (including NMDA receptors) on amphibian developing neuromuscular junction. However, no physiological data support the possible existence and function of these receptors in presynaptic terminals of neurons from the CNS, although such a possibility has been discussed in some electrophysiological studies (see e.g., Manabe et al., 1992; Malgaroli and Tsien, 1992; Wyllie et al., 1994). The astrocyte-evoked AP5-sensitive increase in mPSC frequency after block of postsynaptic NMDA receptors, however, provides experimental support to the idea that NMDA receptors can also be localized in presynaptic terminals of the mammalian CNS.

We have previously demonstrated that astrocyte stimulation reduced the magnitude of action potential-evoked excitatory and inhibitory synaptic currents by decreasing the probability of evoked transmitter release through the activation of presynaptic mGluRs (Araque et al., 1998). This result seems to contrast with the present data showing an increase in the frequency of mPSCs. However, these data can be explained since both phenomena are mediated by separate mechanisms. Indeed, evoked, but not spontaneous transmitter release, depends on Ca^{2+} influx through presynaptic Ca^{2+} channels that can be inhibited by presynaptic mGluRs (Takahashi et al., 1996). Thus, modulation of evoked synaptic transmission is not necessarily associated with changes in the frequency of spontaneous synaptic transmission (e.g., Araque et al., 1994; Gereau and Conn, 1995; Sciancalepore et al., 1995; Fitzsimonds and Dichter, 1996).

In conclusion, we have provided evidence which demonstrates in cell culture that an elevation of Ca^{2+} in astrocytes is both necessary and sufficient to increase the mPSC frequency of adjacent neurons. This astrocyte-induced enhancement of synaptic transmission is AP5-sensitive but is not mediated by postsynaptic NMDA receptors, as indicated by experiments using the open channel blocker of NMDA receptors, MK-801, together with synaptic activation of these receptors. Consequently, we conclude that a Ca^{2+} elevation in astrocytes induces the release of the transmitter glutamate, which causes an NMDA receptor-dependent increase in mPSC frequency by acting on receptors that are

likely present in the presynaptic terminal. Our data reveal new physiological consequences of the proposed existence of bi-directional communication between neurons and astrocytes (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Pasti et al., 1997; Araque et al., 1998; Bezzi et al., 1998) and support a more active role of astrocytes in information processing in the brain.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH (NS24233 and NS37585) and from the Iowa State University Biotechnology Council to P.G.H., and by a postdoctoral fellowship from the Human Frontier Science Program to A.A. The authors would like to thank Prairie Technologies (Waunakee, WI) for a gift of UV transmitting optical fiber and for proprietary knowledge used to construct our fiber photolysis unit.

REFERENCES

- Aoki C, Venkatesan C, Go CG, Mong JA, Dawson TM (1994) Cellular and subcellular localization of NMDA-R1 subunit immunoreactivity in the visual cortex of adult and neonatal rats. *J Neurosci* 14:5202-5222.
- Araque A, Clarac F, Buño W (1994) P-type Ca^{2+} channels mediate excitatory and inhibitory synaptic transmitter release in crayfish muscle. *Proc Natl Acad Sci USA* 91:4224-4228.
- Araque A, Parpura V, Sanzgiri RP, Haydon PG (1998) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur J Neurosci* 10:2129-2142.
- Barria A, Muller D, Derkach V, Griffith LC, Soderling TR (1997) Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. *Science* 276:2042-2045.
- Bekkers JM, Stevens CF (1989) NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus. *Nature* 341:230-233.

- Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Lodi Rizzini B, Pozzan T, Volterra A (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* 391:281-285.
- Bliss TVP, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361:31-39.
- Charles AC (1994) Glia-neuron intercellular calcium signaling. *Dev Neurosci* 16:196-206.
- Charles AC, Dirksen ER, Merrill JE, Sanderson MJ (1993) Mechanisms of intercellular calcium signaling in glial cells studied with dantrolene and thapsigargin. *Glia* 7:134-145.
- Charles AC, Merrill JE, Dirksen ER, Sanderson MJ (1991) Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* 6:983-992.
- Cheramy A, Godeheu G, L'Hirondel M, Glowinski J (1996) Cooperative contributions of cholinergic and NMDA receptors in the presynaptic control of dopamine release from synaptosomes of the rat striatum. *J Pharmacol Exp Ther* 276:616-625.
- Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signalling. *Science* 247:470-473.
- Dani JW, Chernjavsky A, Smith SJ (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* 8:429-440.
- Del Castillo J, Katz B (1954) Quantal components of the endplate potential. *J Physiol (Lond)* 124:560-573.
- Desce JM, Godeheu G, Galli T, Artaud F, Cheramy A, Glowinski J (1992) L-glutamate evoked release of dopamine from synaptosomes of the rat striatum: Involvement of AMPA and NMDA receptors. *Neurosci* 47:333-339.
- Duffy S, MacVicar BA (1995) Adrenergic calcium signaling in astrocyte networks within the hippocampal slice. *J Neurosci* 15:5535-5550.
- Fink K, Bonish H, Gothert M (1990) Presynaptic NMDA receptors stimulate noradrenaline release in the cerebral cortex. *Eur J Pharmacol* 185:115-117.

- Finkbeiner SM (1992) Calcium waves in astrocytes - Filling in the gaps. *Neuron* 8:1101-1108.
- Fitzsimonds RM, Dichter MA (1996) Heterologous modulation of inhibitory synaptic transmission by metabotropic glutamate receptors in cultured hippocampal neurons. *J Neurophysiol* 75:885-893.
- Fu WM, Liou JC, Lee YH, Liou HC (1995) Potentiation of neurotransmitter release by activation of presynaptic glutamate receptors at developing neuromuscular synapses of *Xenopus*. *J Physiol (Lond)* 489:813-823.
- Gereau RW, Conn PJ (1995) Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. *J Neurosci* 15:6879-6889.
- Haley JE, Wilcox GL, Chapman PF (1992) The role of nitric oxide in hippocampal long-term potentiation. *Neuron* 8:211-216.
- Hassinger TD, Atkinson PB, Strecker GJ, Whalen LR, Dudek FE, Koseel AH, Kater SB (1995) Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. *J Neurobiol* 28:159-170.
- Herrero I, Miras-Portugal MT, Sanchez-Prieto J (1992) Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. *Nature* 360:163-166.
- Hessler NA, Shirke AM, Malinow R (1993) The probability of transmitter release at a mammalian central synapse. *Nature* 366:569-572.
- Johnson RR, Jiang X, Burkhalter A (1996) Regional and laminar differences in synaptic localization of NMDA receptor subunit NR1 splice variants in rat visual cortex and hippocampus. *J Comp Neurol* 368:335-355.
- Krebs MO, Trovero F, Desban M, Gauchy C, Glowinski J, Kemel ML (1991b) Distinct presynaptic regulation of dopamine release through NMDA receptors in striosome- and matrix-enriched areas of the striatum. *J Neurosci* 11:1256-1262.
- Liu H, Wang H, Sheng M, Jan LY, Jan YN, Basbaum AI (1994) Evidence for presynaptic N-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn. *Proc Natl Acad Sci USA* 91:8383-8387.

- Malgaroli A, Tsien RW (1992) Glutamate-induced long-term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neurons. *Nature* 357:134-139.
- Manabe T, Renner P, Nicoll RA (1992) Postsynaptic contribution to long-term potentiation revealed by the analysis of miniature synaptic currents. *Nature* 355:50-55.
- Martin D, Bustos GA, Bowe MA, Brady SD, Nadler JV (1991) Autoreceptor regulation of glutamate and aspartate release from slices of the hippocampal CA1 area. *J Neurochem* 56:1647-1655.
- McBain CJ, Dingledine R (1992) Dual-component miniature excitatory synaptic currents in rat hippocampal CA3 pyramidal neurons. *J Neurophysiol* 68:16-27.
- Murphy TH, Blatter LA, Wier WG, Baraban JM (1993) Rapid communication between neurons and astrocytes in primary cortical cultures. *J Neurosci* 13:2672-2679.
- Nedergaard M (1994) Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* 263:1768-1771.
- Newman EA, Zahs KR (1997) Calcium waves in retinal glial cells. *Science* 275:844-847.
- Nicoll RA, Malenka RC (1995) Contrasting properties of two forms of long-term potentiation in the hippocampus. *Nature* 377:115-118.
- Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte-neuron signalling. *Nature* 369:744-747.
- Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes *in situ*. *J Neurosci* 17:7817-7830.
- Pittaluga A, Raiteri M (1992) N-methyl-D-aspartic acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. I. Localization on axon terminals and pharmacological characterization. *J Pharmacol Exp Ther* 260:232-237.
- Porter JT, McCarthy KD (1996) Hippocampal astrocytes *in situ* respond to glutamate released from synaptic terminals. *J Neurosci* 16:5073-5081.
- Reid CA, Clements JD, Bekkers JM (1997) Nonuniform distribution of Ca^{2+} channel subtypes on presynaptic terminals of excitatory synapses in hippocampal cultures. *J Neurosci* 17:2738-2745.

- Rosenmund C, Clements JD, Westbrook GL (1993) Nonuniform probability of glutamate release at a hippocampal synapse. *Science* 262:754-757.
- Schuman EM, Madison DV (1991) A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* 254:1503-1506.
- Sciancalepore M, Stratta F, Fisher ND, Cherubini E (1995) Activation of metabotropic glutamate receptors increase the frequency of spontaneous GABAergic currents through protein kinase A in neonatal rat hippocampal neurons . *J Neurophysiol* 74:1118-1122.
- Siegel SJ, Brose N, Janssen WG, Gasic GP, Jahn R, Heinemann SF (1994) Regional, cellular and ultrastructural distribution of N-methyl-D-aspartate receptor subunit 1 in monkey hippocampus. *Proc Natl Acad Sci USA* 91:564-568.
- Takahashi T, Forsythe ID, Tsujimoto T, Barnes-Davies M, Onodera K. (1996) Presynaptic calcium current modulation by a metabotropic glutamate receptor. *Science* 274:594-597.
- Van Bockstaele EJ, Colago EE (1996) Selective distribution of the NMDA-R1 glutamate receptor in astrocytes and presynaptic axons terminals in the nucleus locus coeruleus of the rat brain: an immunoelectron microscopic study. *J Comp Neurol* 369:483-496.
- Wang JKT, Thukral V (1996) Presynaptic NMDA receptors display physiological characteristics of homomeric complexes of NR1 subunits that contain the exon 5 insert in the N-terminal domain. *J Neurochem* 66:865-868.
- Wyllie DA, Manabe T, Nicoll RA (1994) A rise in postsynaptic Ca^{2+} potentiates miniature excitatory postsynaptic currents and AMPA responses in hippocampal neurons. *Neuron* 12:127-138.
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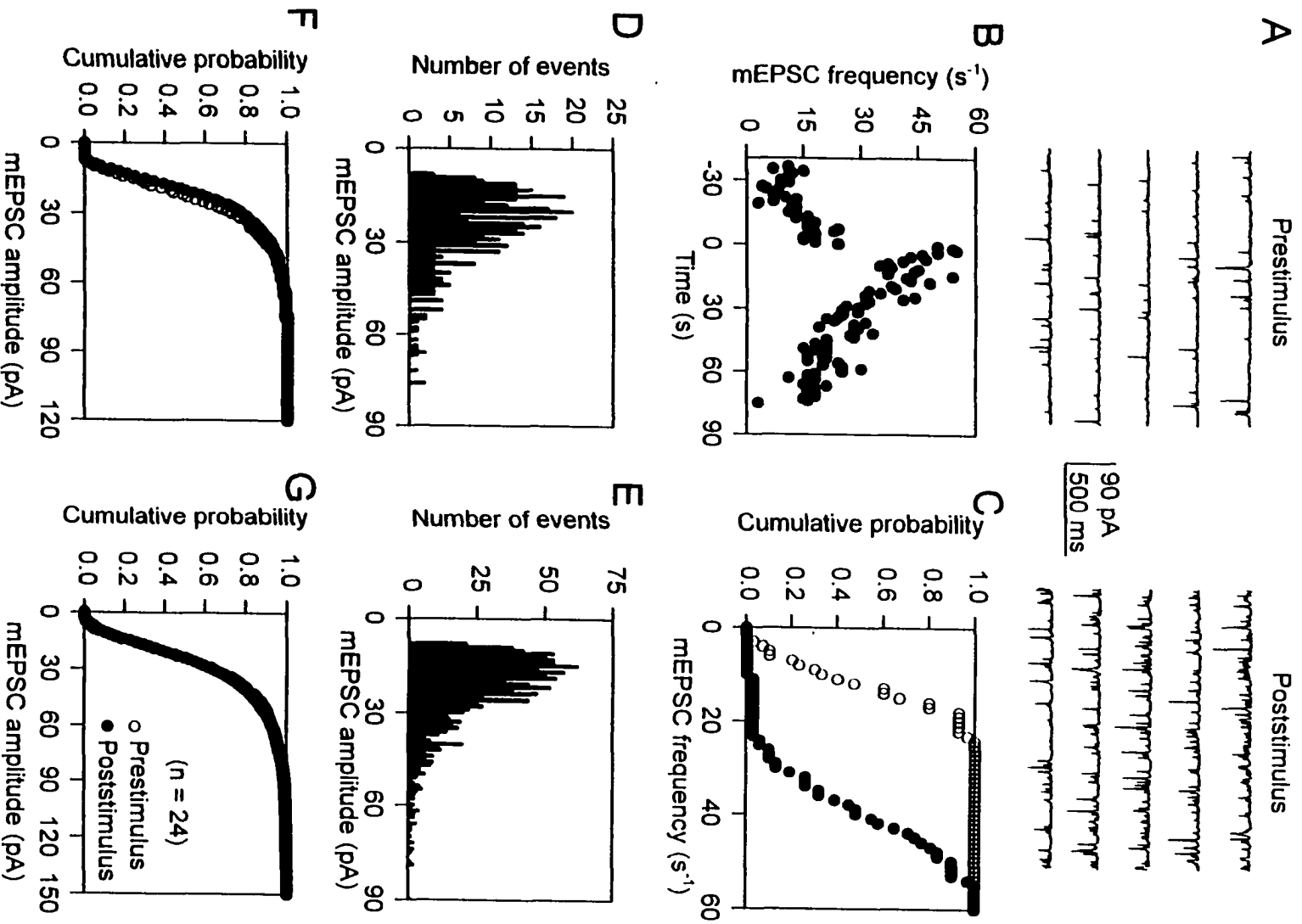


Figure 1

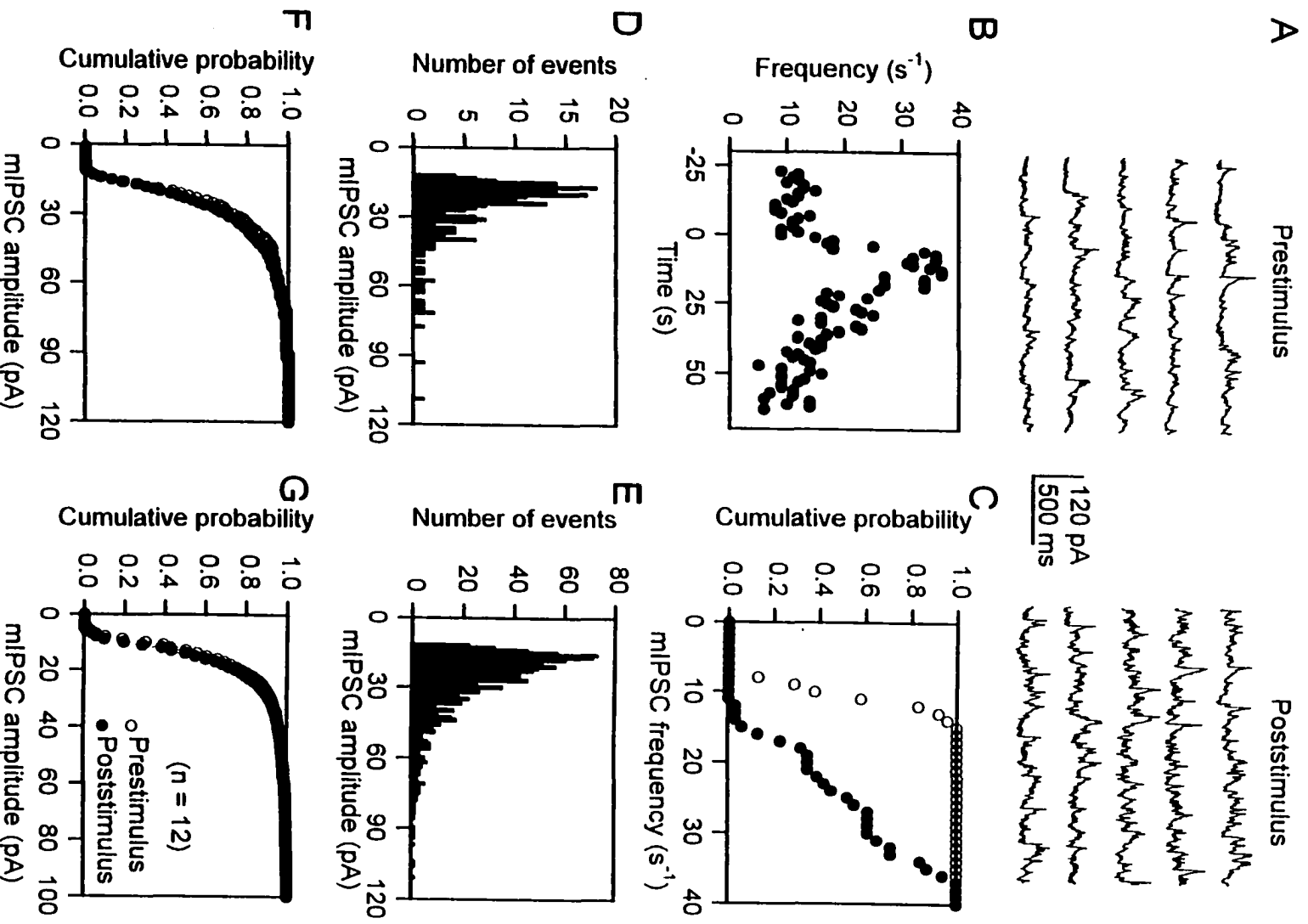
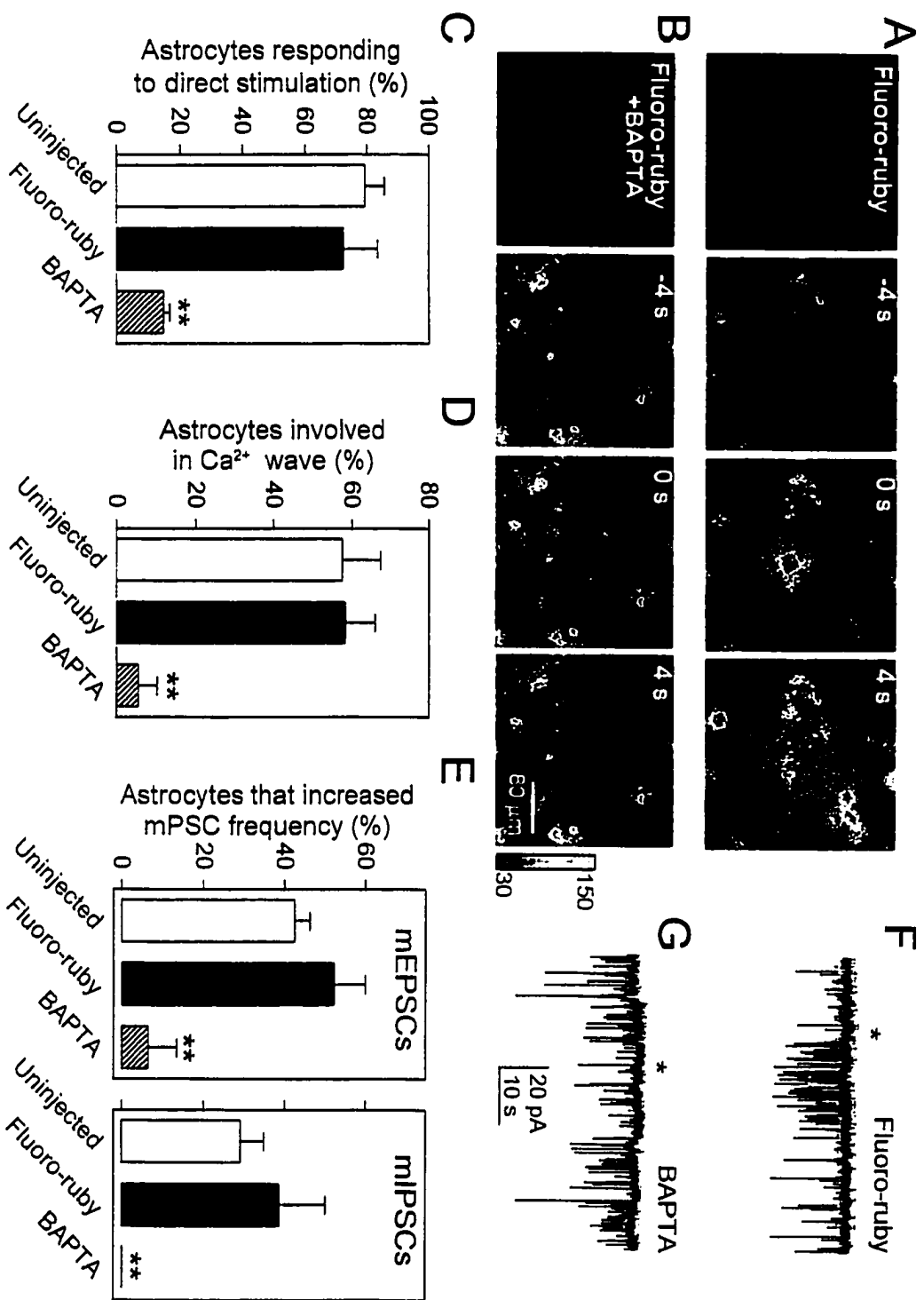


Figure 2



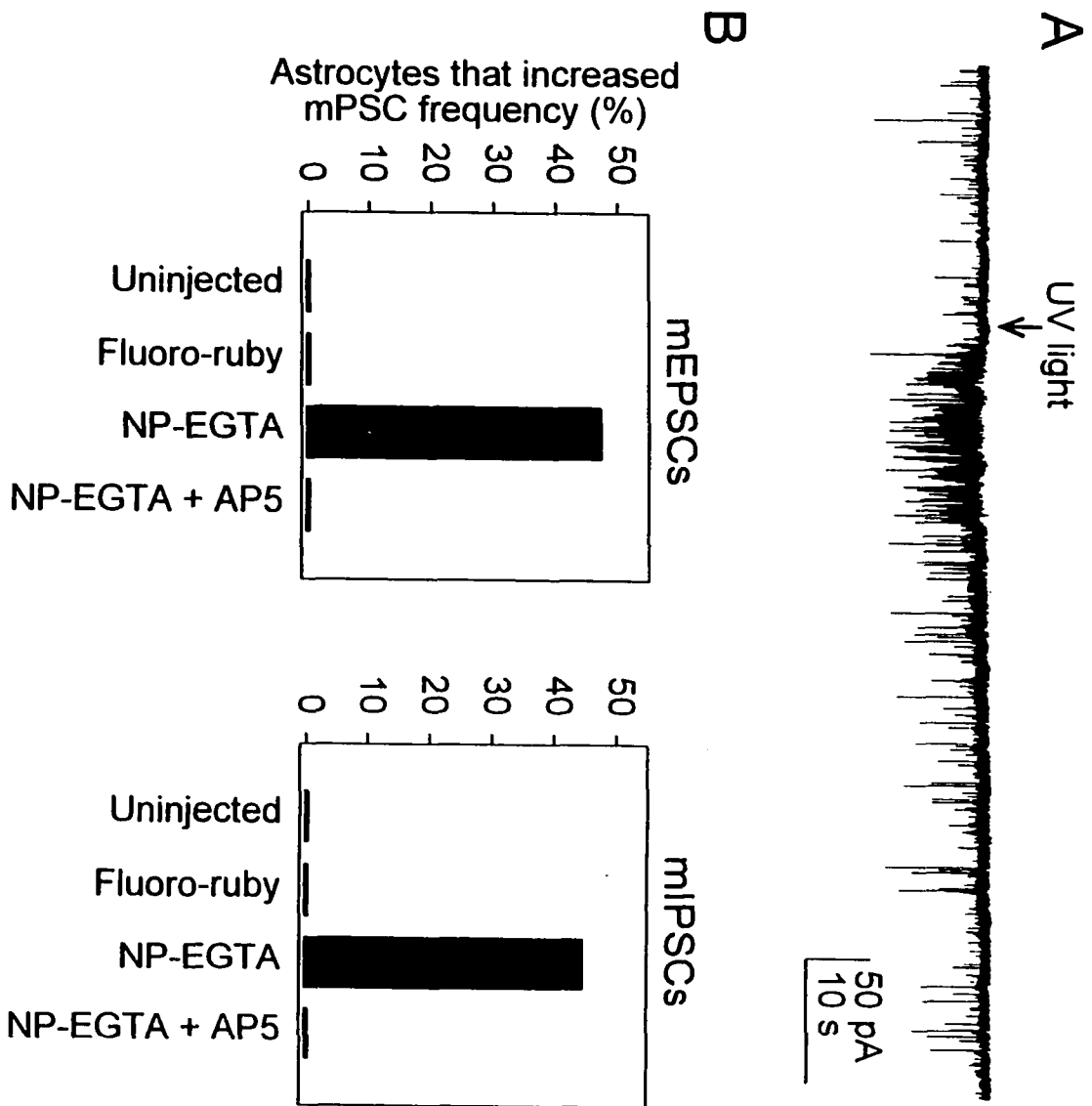


Figure 4

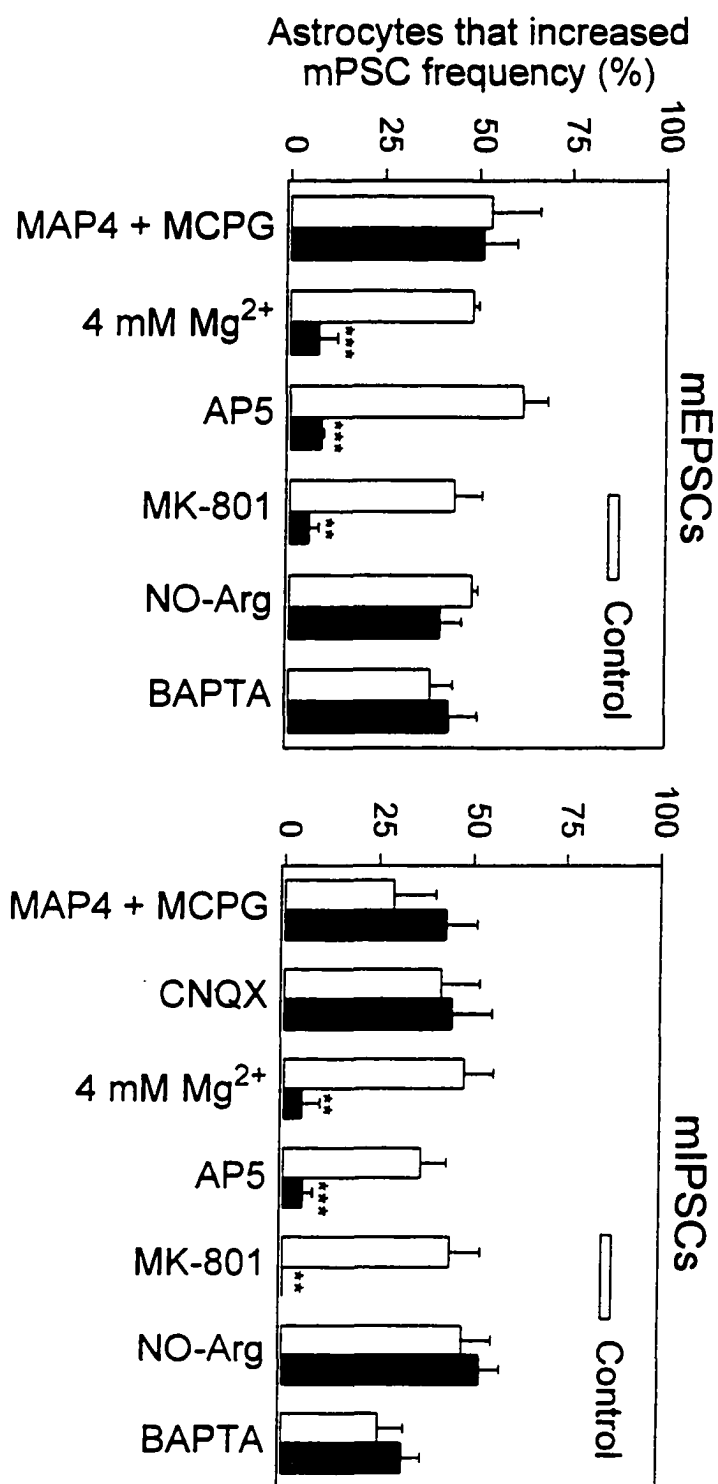


Figure 5

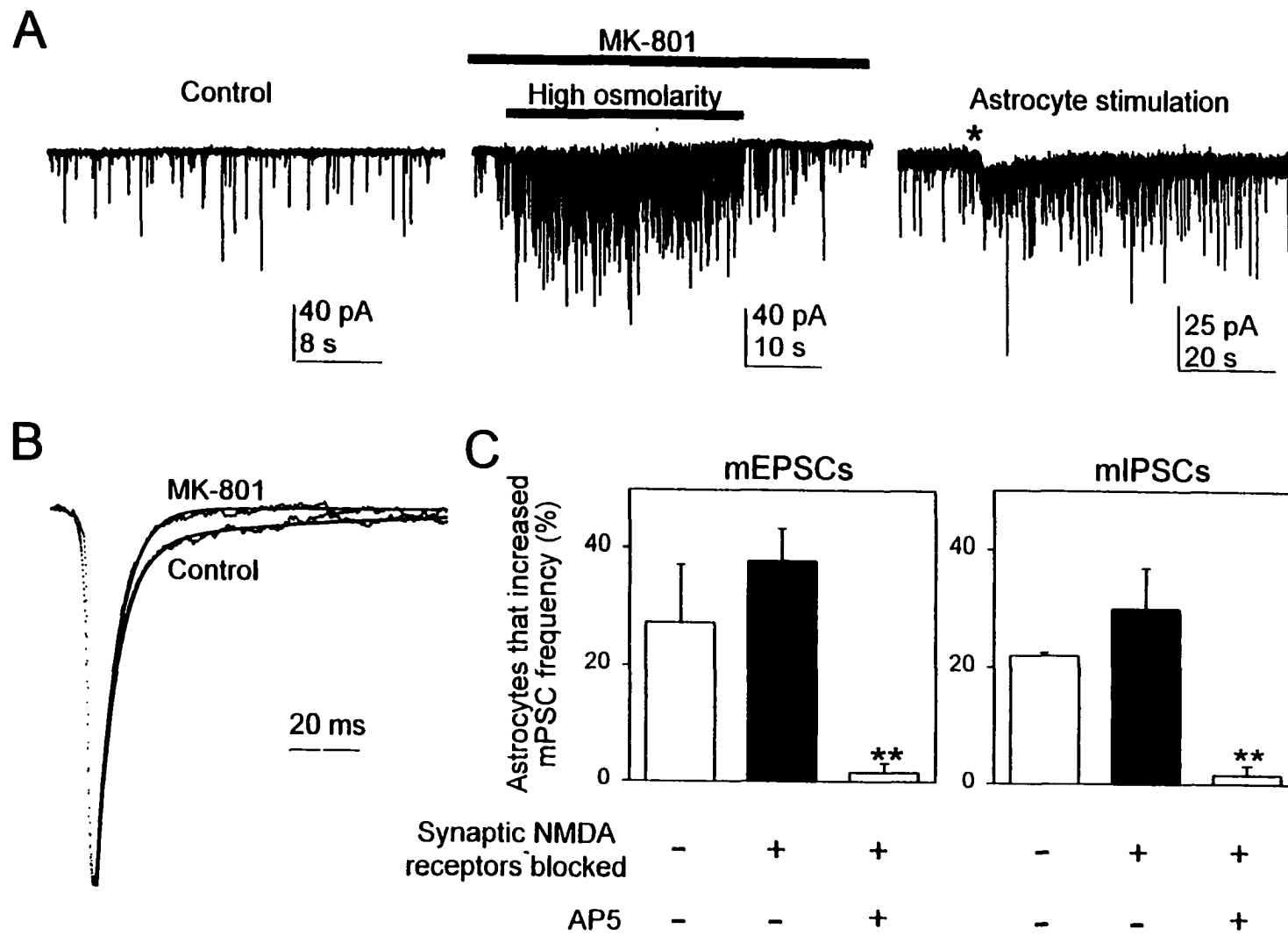


Figure 6

FIGURE LEGENDS

FIGURE 1. Astrocyte stimulation increases mEPSC frequency. *A*, mEPSCs recorded at a holding potential of -60 mV before and after mechanical stimulation of an astrocyte. *B*, Time course of the mEPSC frequency calculated in 1 s bins. Zero time corresponds to the time of astrocyte stimulation. *C*, Cumulative probability plot of the mEPSC frequency 30 s before and after astrocyte stimulation (open and filled symbols, respectively). *D* and *E*, Histograms of mEPSC amplitudes (bin width: 1 pA) recorded 30 s before and after astrocyte stimulation, respectively. *F*, Cumulative probability plot of the mEPSC amplitudes recorded 30 s before and after astrocyte stimulation (open and filled symbols, respectively). *G*, Average ($n = 24$) cumulative probability plot of the mEPSC amplitudes recorded 30 s before and after astrocyte stimulation (open and filled symbols, respectively). Bars showing SEM are smaller than symbol size. To obtain cumulative probability plots, the frequency and the amplitudes of mEPSCs were calculated in 1 s and 1 pA bins, respectively.

FIGURE 2. Astrocyte stimulation increases mIPSC frequency. *A*, mIPSCs recorded at a holding potential of -30 mV before and after mechanical stimulation of an astrocyte. *B*, Time course of the mIPSC frequency calculated in 1 s bins. Zero time corresponds to the time of astrocyte stimulation. *C*, Cumulative probability plot of the mIPSC frequency 30 s before and after astrocyte stimulation (open and filled symbols, respectively). *D* and *E*, Histograms of mIPSC amplitudes recorded 30 s before and after astrocyte stimulation, respectively. *F*, Cumulative probability plot of the mIPSC amplitudes recorded 30 s before and after astrocyte stimulation (open and filled symbols, respectively). *G*, Average ($n = 12$) cumulative probability plot of the mEPSC amplitudes recorded 30 s before and after astrocyte stimulation (open and filled symbols, respectively). Cumulative probability plots were obtained as in Figure 1.

FIGURE 3. Microinjection of the Ca^{2+} chelator BAPTA into an astrocyte prevents the propagation of astrocyte Ca^{2+} waves and blocks the astrocyte-induced increase in mPSC

frequency. *A*, cultures were loaded with the Ca^{2+} indicator fluo-3 to monitor the stimulus-induced Ca^{2+} elevations in astrocytes, and a single astrocyte was microinjected with fluoro-ruby (left panel). Right panels show images in pseudocolor mode representing intensity of fluo-3 emission, taken before, during and after mechanical stimulation of the fluoro-ruby injected cell, at the times indicated. Zero time corresponds to the time of astrocyte stimulation. Mechanical stimulation increases intracellular Ca^{2+} in the injected cell as well as in neighboring unstimulated astrocytes. *B*, as in *A* but with a single astrocyte microinjected with fluoro-ruby and BAPTA (left panel). Mechanical stimulation of the injected cell did not change the fluorescent emission of fluo-3 either in the stimulated or neighboring astrocytes. *C* and *D* show quantitative data taken from these experiments. The number of astrocytes involved in Ca^{2+} waves was quantified by the proportion of non-stimulated cells within the field of view which responded with a Ca^{2+} elevation. While the ability of astrocytes to respond to direct stimulation or to evoke Ca^{2+} waves (*C* and *D*, respectively) was unaffected by the injection of fluoro-ruby, it was significantly reduced by BAPTA-injection. In parallel studies mPSCs were recorded in response to mechanical stimulation of astrocytes. While the astrocyte-induced mPSC frequency increase was not affected by injection of fluoro-ruby (*E* and *F*), it was prevented by injection of BAPTA (*E* and *G*). (** represents $p < 0.01$).

FIGURE 4. Ca^{2+} elevation in astrocytes is sufficient to increase the frequency of mPSCs. *A*, whole cell recording from a neuron adjacent to an astrocyte that had been microinjected with the UV sensitive Ca^{2+} -cage NP-EGTA. UV photolysis (arrow) increased the Ca^{2+} level in the astrocyte and caused an increase in the frequency of mEPSCs. *B*, graphs summarizing the effects of photolysis on the mEPSC and mIPSC frequency. Pulses of UV light only increased the frequency of mPSCs when the astrocyte was injected with NP-EGTA. While the frequency of mPSCs was not modified by UV stimulation of uninjected astrocytes (0 out of 6 cells) or astrocytes injected with fluoro-ruby alone (0 out of 22 cells), photolysis of NP-EGTA-injected astrocytes increased the frequency of mEPSCs (8 out of 17 cells) and mIPSCs (4 out of 9 cells). This photolysis-dependent increase in mPSC

frequency was prevented by incubation with 50 μ M AP5 (0 out of 12 NP-EGTA-injected astrocytes).

FIGURE 5. Astrocyte-induced increase in mPSC frequency is mediated by NMDA receptors. Percentage of mechanically stimulated astrocytes that increased the frequency of mEPSCs (left) and mIPSCs (right) in 0.5 mM MAP4 and 0.5 mM MCPG, 10 μ M CNQX, 4 mM Mg^{2+} , 50 μ M AP5, 50 μ M NO-Arg, and after dialysis of the postsynaptic cell with BAPTA (10 mM, in the recording pipette), and in their respective control solution in parallel cultures. Significant differences with respect to control were established by the Student's t-test at $p < 0.01$ (**) $p < 0.001$ (***).

FIGURE 6. AP5-sensitive astrocyte-induced increase in mPSC frequency is not mediated by postsynaptic NMDA receptors. *A*, mPSCs recorded at a holding potential of -60 mV in control solution (left). To block postsynaptic NMDA receptors, MK-801 was added to the saline while synaptic release of glutamate was stimulated by pressure-ejection of high osmolarity saline (center). Several 1-50 s duration pressure pulses of high osmolarity solution (obtained by addition of 0.3 M sucrose to the standard saline) were delivered, but only one is shown. Right panel shows mPSCs recorded at a holding potential of -30 mV following blockage of postsynaptic NMDA receptors with MK-801. The trace has been offset for illustration purposes. Mechanical stimulation of the astrocyte is indicated by the asterisk. Note that despite the selective block of postsynaptic NMDA receptors, stimulation of astrocytes still evoked an increase in frequency of mPSCs. *B*, Averaged ($n > 50$) mEPSCs (dotted lines) in control solution and in MK-801 after several pressure pulses of high osmolarity solution. Synaptic activation with high osmolarity saline blocked postsynaptic NMDA receptors because mEPSCs now exhibit only one time constant of decay. The decay time course of mEPSCs were fitted to two and to a single exponential function in control and following MK-801 treatment, respectively (continuous lines). *C*, Proportion of astrocytes in which mechanical stimulation evoked an increase in the

frequency of mEPSCs (left) and mIPSCs (right) in control solution, and after exposure to MK-801 in the absence and the presence of 50 μ M AP5 . (** represents $p < 0.01$).

CHAPTER 4. PROSTAGLANDIN E₂ EVOKES GLUTAMATE-DEPENDENT ASTROCYTE-NEURON SIGNALING IN HIPPOCAMPAL CELL CULTURES

A paper submitted to the Journal of Neuroscience

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ABSTRACT

Recent Ca²⁺ imaging studies in cell culture and *in situ* have shown that Ca²⁺ elevations in astrocytes stimulate glutamate release and increase neuronal Ca²⁺ levels (Parpura et al., 1994; Pasti et al., 1997), and that this astrocyte-neuron signaling can be evoked by prostaglandin E₂ (PGE₂) (Bezzi et al., 1998). Araque et al. (1998a,b) have demonstrated that mechanical or electrical stimulation of astrocytes raises their internal Ca²⁺ levels and evokes a glutamate-mediated slow inward current (SIC) in adjacent neurons. We investigated the electrophysiological consequences of the PGE₂-mediated astrocyte-neuron signaling using whole-cell recordings on cultured rat hippocampal cells. We found that focal application of PGE₂ to astrocytes evoked a Ca²⁺ wave in neighboring astrocytes and a SIC in the adjacent neurons. This neuronal response was mediated through both NMDA and non-NMDA glutamate receptors and remained unaffected by the metabotropic glutamate receptor antagonists. The neuronal SIC required the presence of an astrocyte Ca²⁺ wave and the amplitude of the current was determined by the magnitude of the wave. Our results provide electrophysiological evidence for ligand-induced astrocyte-neuron signaling and confirm our hypothesis that astrocyte-neuron signaling is associated with glutamate-dependent currents in adjacent neurons.

INTRODUCTION

During the last decade our understanding of glial cells and their role in the central nervous system (CNS) has changed significantly (For reviews see, Barres, 1991; Smith, 1994; Parpura et al., 1996; Ransom and Orkand, 1996; Theodosis and MacVicar, 1996). Previously it was thought that direct intercellular signaling in the CNS is the exclusive domain of neurons, while glia pursue a more passive role of merely providing structural, trophic and metabolic support to neurons. A number of new studies have prompted a reexamination of this traditional view and astrocytes, a subtype of glial cells, have emerged as cells that can participate in bi-directional intercellular signaling with neurons (Dani et al., 1992; Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Porter and McCarthy, 1996; Pasti et al., 1997; Bezzi et al., 1998; Araque et al., 1998a,b; Newman and Zahs, 1998). Astrocytes communicate among themselves based on variations in their cytosolic Ca^{2+} concentration, which can propagate as Ca^{2+} waves through the network of neighboring astrocytes (Cornell-Bell et al., 1990; Charles et al., 1991; Finkbeiner, 1992; Dani et al., 1992; Verkhratsky and Kettenmann, 1996). Neuronal activity can trigger Ca^{2+} waves in astrocytes, both *in vitro* (Dani et al., 1992) and *in situ* (Porter and McCarthy, 1996) indicating that neurons can signal to astrocytes. Finally, the possibility that astrocytes may in turn signal back to neurons has been supported by several studies showing that stimulation of astrocytes leads to glutamate receptor-dependent Ca^{2+} elevations in adjacent neurons (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Pasti et al., 1998; Bezzi et al., 1998; Araque et al., 1998a,b; Newman and Zahs, 1998).

Prostaglandins have been implicated in the astrocyte-induced glutamate-mediated neuronal Ca^{2+} elevations (Bezzi et al., 1998). It has been suggested that coactivation of the AMPA/kainate and metabotropic glutamate receptors (mGluR) on astrocytes stimulated glutamate release from these cells through a Ca^{2+} -dependent process mediated by prostaglandins. Prostaglandins are derived from arachidonic acid by the action of the enzyme prostaglandin endoperoxidase H synthase, also referred to as cyclooxygenase. Pharmacological inhibition of the synthesis of arachidonic acid or the activity of

cyclooxygenase prevented glutamate release from astrocytes whereas application of prostaglandins, in particular prostaglandin E_2 (PGE_2), promoted Ca^{2+} -dependent glutamate release from astrocytes. Consequently, glutamate receptor-dependent Ca^{2+} increases were observed in the adjacent neurons, in cell cultures and in acute brain slices (Bezzi *et al.*, 1998).

We investigated the electrophysiological consequences of the astrocyte-neuron signaling evoked by the neuroligand PGE_2 . Recently, Araque *et al.* (1998a,b) provided electrophysiological evidence for the existence of astrocyte-neuron signaling. They showed that mechanical or electrical stimulation of an astrocyte gives rise to an intercellular Ca^{2+} wave among astrocytes which then evokes 1) a slow inward current (SIC) in adjacent neurons through activation of N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors and 2) an NMDA receptor-dependent increase in the frequency of the miniature postsynaptic currents (mPSCs) (Araque *et al.*, 1998a,b). Their results demonstrated that astrocytes modulate neuronal currents and synaptic transmission in culture.

However, it still remains to be seen whether a biologically relevant stimulus for astrocytes, such as receptor activation by an endogenous ligand, could evoke this astrocyte-neuron signaling in adjacent neurons. To address this question, we investigated the physiological consequences of PGE_2 -induced elevations in astrocyte Ca^{2+} . Here we demonstrate that focal application of PGE_2 evokes Ca^{2+} elevations in astrocytes which lead to glutamate-mediated currents in adjacent neurons. This SIC is mediated through NMDA and non-NMDA glutamate receptors and is not affected by metabotropic glutamate receptor antagonists.

MATERIALS AND METHODS

Culture preparation

Primary cultures of mixed hippocampal neurons and astrocytes from 1- to 3-day-old postnatal rats were prepared as previously described (Araque *et al.*, 1998a) and were used after 8-25 days in culture.

Electrophysiology

Whole-cell patch clamp recordings were obtained from neurons with an Axopatch-1C amplifier and pClamp software (Axon Instruments, Foster City, CA). External control solution contained (in mM): 140 NaCl, 5 KCl, 4 CaCl₂, 10 HEPES, 10 Glucose, 6 Sucrose (pH 7.35). Since Araque et al. (1998a) suggested that SIC is partly mediated through NMDA glutamate receptors, experiments were performed in the presence of 10 μ M glycine and 0 Mg²⁺ to achieve maximal NMDA receptor activation. To record mPSCs, neuronal action potentials were blocked by addition of 1 μ M tetrodotoxin (TTX) to the bath. The patch pipette solution contained (in mM): 140 K-gluconate, 10 EGTA, 4 Mg-ATP, 0.2 Tris-GTP, and 10 HEPES (pH 7.35). The membrane potential was held at -60 mV. For thapsigargin treatment, cells were incubated with 1 μ M thapsigargin for 60 minutes.

Neurons were differentiated from astrocytes based on their morphology. This morphological identification of neurons was further confirmed electrophysiologically by the presence of TTX sensitive Na⁺ currents. Confluent astrocytes < 200 μ m from the soma of the recorded neuron, were stimulated by focal application of 1mM PGE₂ in 1% ethanol (pipette concentration) by pressure ejection (5s, 20-25 p.s.i) (Narishige IM-200, Narishige, Greenvale, NY) of PGE₂ from a micropipette. Positioning of micropipette was controlled by an Eppendorf micro-manipulator. At least six astrocytes were stimulated in each parallel control and test condition and data were obtained from at least three different experiments (i.e., at least 18 astrocytes stimulated in each condition). Only currents \geq 10 pA were considered PGE₂-evoked SIC, i.e., at least three times higher than the standard deviation of the baseline noise. The incidence of astrocyte-induced responses was defined as the proportion of responses relative to the total number of astrocytes stimulated in each experiment, and statistical differences were established using the Student's t-test. All experiments were performed at room temperature (20-23 °C). Data are expressed as mean \pm SEM.

Ca²⁺ measurements

Ca²⁺ levels in astrocytes were monitored by fluorescence microscopy using the Ca²⁺ indicator fluo-3. Cultures were incubated at 37 °C for 45 minutes with the acetoxymethyl ester of fluo-3 (fluo3-AM, 10 µg/ml; Molecular Probes, Eugene, OR). After washing, the indicator was allowed to de-esterify for 45 minutes. Fluo-3 loaded cells were visualized using a IC-300 intensified CCD camera (Photon Technology International, Monmouth Junction, NJ) or a silicon intensified target (SIT) camera (Hamamatsu Photonic System, Bridgewater, NJ) attached to a Nikon diaphot inverted microscope. Quantitative fluorescence measurements were made using the NeD_{LC} video software (Prairie Technologies, LLC; Waunakee, WI). For purposes of data analysis, a Ca²⁺ wave was defined as a 30% increase in signal intensity over baseline ($\Delta F/F_0$), that spread from the stimulated cell to at least one more adjacent cell. The wave radius was estimated by calculating the average distance traveled by the wave in six different directions with the stimulated cell at the center.

Microinjection into astrocytes

In some experiments we injected the Ca²⁺ chelator BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; tetrapotassium salt) into individual astrocytes as described elsewhere (Araque et al., 1998a,b). Microinjection pipettes (tip diameter of ~ 400 nm) were pulled from Kwik-Fil borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) using a Sutter P-2000 micropipette puller (Sutter Instrument Co., Novato, CA). Pipettes were filled with a solution containing 375 mM BAPTA (pH 7.2) and 0.25 mM fluoro-ruby. Since, fluoro-ruby contains a dextran moiety which prevents its passage through gap junctions, the dye is retained within the injected cell allowing identification of injected cells. This solution was pressure injected into single astrocytes using a 300 ms, 15 p.s.i pulse (Narishige IM-200, Narishige, Greenvale, NY). After injections cells were allowed to recover for 30 minutes before performing electrophysiology or Ca²⁺ imaging experiments. Positioning of micropipettes for injection was controlled by an Eppendorf micro-manipulator. Based on quantification of the fluoro-

ruby fluorescence, the resulting final intracellular BAPTA concentration was estimated to be 1-2 mM (Araque et al., 1998a).

RESULTS

Focal application of PGE₂ to astrocytes evokes a Ca²⁺ wave among astrocytes and a SIC in adjacent neurons

Astrocyte-neuron signaling was studied using postnatal rat hippocampal cultures consisting of neurons and astrocytes. We first determined the ability of PGE₂ to induce Ca²⁺ elevations in astrocytes. Ca²⁺ elevations were monitored using the fluorescent Ca²⁺ indicator, fluo-3. Focal application of PGE₂ to astrocytes successfully induced Ca²⁺ waves through the astrocytic network. (64.5 ± 3.6% of PGE₂ applications resulted in an interastrocyte wave, average of four experiments). Application of PGE₂ caused a Ca²⁺ elevation in the stimulated cell ($\Delta F/F_0 = 85.6 \pm 9.04$, n = 21), which within 1-2 seconds spread to neighboring astrocytes as a nondecremental, regenerative Ca²⁺ wave (figure 1A). Most of these Ca²⁺ waves spread in a radial manner with a mean radius of 94.9 ± 1.7 microns (The astrocytes were usually 30-50 μ m in diameter). Application of the carrier solution alone (1% ethanol in external saline) did not cause Ca²⁺ elevations in astrocytes (0.0%, n = 6), indicating that the increases in the Ca²⁺ levels of astrocytes were not due to nonspecific effects of the carrier or stimulus itself, but rather specifically due to application of PGE₂.

To investigate the electrophysiological effects of PGE₂-induced astrocyte Ca²⁺ waves on adjacent neurons, we recorded whole cell currents from adjacent neurons. PGE₂ was focally applied to an astrocyte, while responses were detected from adjacent (<200 μ m) neurons in the whole-cell recording mode. In 51.7 ± 3.5 % cases, PGE₂-evoked stimulation of astrocytes elicited a SIC in adjacent neurons (figure 1B). The currents originated 8.9 ± 1.9 s after stimulation, slowly reached their maximum amplitude (32.0 ± 2.5 pA) and lasted for several seconds (12.6 ± 1.0 s, n = 33). In addition to this response, PGE₂ application resulted in a transient increase in the frequency of miniature postsynaptic currents (figure

IC), in 7 out of 24 cells, which agrees with the previous reports of Araque et al. (1998a,b). For the purpose of this study, we focused our efforts on the analysis of the SIC.

To control for any inadvertent mechanical stimulation of astrocytes during focal application of PGE₂ and to determine whether the SIC is indeed due to application of PGE₂, we made focal application to astrocytes of the carrier alone while recording from adjacent neurons. Application of the carrier did not elicit a SIC in neurons ($P < 0.01$, three experiments) (Figure 1B, 2A), confirming that the SIC is specifically induced by PGE₂.

PGE₂-evoked SIC is mediated by NMDA and non-NMDA glutamate receptors

A number of studies have indicated that the astrocyte-evoked neuronal Ca²⁺ elevations are mediated by glutamate released from astrocytes (Parpura et al., 1994; Hassinger et al., 1995; Pasti et al., 1998; Bezzi et al., 1998; Araque et al., 1998a,b, b; Newman and Zahs 1998). Consistent with this finding, Araque et al. (1998a,b) reported that the neuronal SIC evoked by mechanical or electrical stimulation of astrocytes, was sensitive to NMDA and non-NMDA glutamate receptor antagonists. To investigate whether the PGE₂-evoked SIC has a similar underlying mechanism, we studied its pharmacology.

Pharmacological studies showed that the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20μM) reduced the incidence of the SIC ($51.7 \pm 3.5\%$ in control and $13.4 \pm 1.7\%$ in the presence of CNQX, $P < 0.01$). A similar reduction in the incidence of the SIC was observed in the presence of D-2-amino-5-phosphonopentanoic acid (D-AP5; 50μM), an NMDA glutamate receptor antagonist ($18.5 \pm 1.8\%$, $P < 0.01$). The combination of CNQX and D-AP5 together, practically abolished the SIC ($3.7 \pm 3.7\%$, $P < 0.001$) (Figure 2A). The mGluR antagonists (S)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4; 0.5mM) and (S)-α-methyl-4-carboxy-phenyl-glycine (MCPG; 0.5mM), did not alter the incidence or the amplitude of the PGE₂-evoked SIC (Figure 2A). Taken together, these results indicate that the PGE₂-evoked SIC is mediated by activation of both NMDA and non-NMDA receptors, but not by metabotropic glutamate receptors.

PGE₂-mediated astrocyte-neuron signaling requires the presence of astrocyte Ca²⁺ waves

While the pharmacological experiments support the notion that PGE₂ causes glutamate-dependent neuronal currents, it is unclear whether PGE₂ acts directly on neurons, rather than through the Ca²⁺-dependent signaling pathway of astrocytes. To distinguish between these two possibilities we developed experimental approaches which selectively block the PGE₂-induced astrocyte Ca²⁺ elevations. First, we blocked the propagation of Ca²⁺ waves within astrocytes by application of thapsigargin. Thapsigargin depletes the internal Ca²⁺ stores required for propagation of the wave, by inhibiting the Ca²⁺-ATPase of the endoplasmic reticulum (Charles 1993; Araque *et al.*, 1998a,b). In astrocytes loaded with the Ca²⁺-indicator fluo-3, application of thapsigargin completely suppressed PGE₂-evoked Ca²⁺ waves among astrocytes ($P < 0.001$) (control 63.8 ± 11.3 %, thapsigargin, 0.0 ± 0.0 %) (Figure 3A, B, D). Interestingly, along with blocking propagation of Ca²⁺ waves, thapsigargin also prevented a Ca²⁺ elevation in the stimulated cell (Figure 3C), suggesting that the PGE₂-evoked Ca²⁺ elevation in astrocytes requires mobilization of Ca²⁺ from internal stores (control 71.6 ± 10.1 %, thapsigargin 4.2 ± 4.2 %) (Figure 3C).

Next, we asked whether blocking Ca²⁺ waves in astrocytes with thapsigargin, also blocks the neuronal responses. Cells were treated with thapsigargin, as described above, and whole-cell currents were recorded from neurons while stimulating astrocytes. Incubation with thapsigargin significantly reduced the incidence of the PGE₂-evoked SIC (77.8 ± 14.7 % in control and 12.2 ± 6.2 % in thapsigargin, $P < 0.01$) (Figure 3E). Taken together, these results are consistent with the idea that the presence of a Ca²⁺ wave in astrocytes is required for the PGE₂-evoked neuronal SIC.

Ca²⁺ elevation specifically in astrocytes is necessary for PGE₂-evoked astrocyte-neuron signaling

The outcome of these experiments is consistent with the hypothesis that the SIC requires Ca²⁺ elevations in astrocytes, although the possibility that thapsigargin may have acted through neuronal Ca²⁺ stores cannot be discounted. To specifically block Ca²⁺

signaling only in astrocytes, we microinjected the Ca^{2+} chelator BAPTA directly into a single astrocyte. To mark the injected cell, the fluorescent dye, fluoro-ruby, was included in the injection solution. After 30 minutes, we assessed whether the microinjections impaired the ability of astrocytes to respond to PGE_2 . As shown in Figure 4A, application of PGE_2 to a fluoro-ruby injected cell, initiated a propagating Ca^{2+} wave, indicating that the fluoro-ruby injected astrocytes are capable of responding to PGE_2 . Stimulation of an uninjected astrocyte or an astrocyte injected with fluoro-ruby resulted in the stimulated cell responding with a Ca^{2+} elevation which spread as a propagating Ca^{2+} wave (uninjected, 4 out of 7 cells; fluoro-ruby injected, 3 out of 6 cells). In contrast, injection of BAPTA (figure 4B), prevented Ca^{2+} elevations in the injected cells (1 out of 7 cells) as well as the propagation of Ca^{2+} wave among astrocytes after PGE_2 application (0 out of 7 cells). These results are summarized in Figure 4C and D.

After we established that microinjection of the Ca^{2+} chelator BAPTA into astrocytes selectively blocks Ca^{2+} waves among astrocytes, we investigated whether they are necessary for the PGE_2 -evoked neuronal SIC. Astrocytes were injected with either fluoro-ruby alone, or together with BAPTA. Injected cells were stimulated by PGE_2 application, while neuronal recordings were obtained under voltage-clamp conditions. Stimulation of astrocytes injected with fluoro-ruby alone, elicited neuronal SICs in $69.4 \pm 19.4\%$ of the cases as compared to $58.9 \pm 14.4\%$ in uninjected cells (Figure 4E), confirming that microinjections *per se* did not affect the ability of astrocytes to evoke a PGE_2 -mediated SIC. Injection of BAPTA practically abolished the occurrence of a SIC ($9.5 \pm 9.5\%$, $P < 0.01$) (Figure 4E). In summary, these experiments show that microinjection of the Ca^{2+} chelator BAPTA into a specific astrocyte, suppresses Ca^{2+} elevations in the injected astrocyte, blocks initiation and propagation of the Ca^{2+} wave and consequently prevents the appearance of the glutamate receptor-dependent SIC in adjacent neurons. These results demonstrate that the PGE_2 effects are mediated through astrocytes, not by neurons and that an elevation in the astrocyte Ca^{2+} is necessary for evoking the neuronal response.

Comparison between mechanically evoked SIC and PGE₂-evoked SIC

While performing the Ca²⁺ imaging experiments to evaluate the effect of PGE₂ application on astrocytes, it became apparent that the PGE₂-induced Ca²⁺ waves in astrocytes were smaller than those seen previously with a mechanical stimulation (Araque et al. 1998a,b). A careful analysis of the Ca²⁺ waves induced by a mechanical stimulation as opposed to PGE₂ application revealed that the ability of PGE₂ to induce Ca²⁺ waves differed significantly from the mechanical stimulation ($P < 0.05$) (Figure 5A). The Ca²⁺ waves induced by mechanical stimulation propagated over a larger distance than those by PGE₂ stimulation (mean radius, mechanically-evoked Ca²⁺ wave = $172.68 \pm 8.54 \mu\text{m}$, PGE₂-evoked Ca²⁺ wave = $94.95 \pm 7.81 \mu\text{m}$, $P < 0.001$) (Figure 5C). Once we established that the Ca²⁺ waves induced by PGE₂ in astrocytes were smaller compared to those induced mechanically, we investigated whether the SIC evoked by the two stimuli also differed correspondingly. We found that consistent with the differential abilities of the two stimuli to evoke Ca²⁺ waves, the ability to evoke SIC also differed significantly ($P < 0.05$). The amplitude of the SIC elicited by PGE₂ was significantly smaller ($155.79 \pm 16.63 \text{ pA}$ with mechanical stimulation, 32.01 ± 5.11 with focal application of PGE₂, $P < 0.001$) (Figure 5E). These results suggest that the neuronal SIC is in direct response to the Ca²⁺ elevation in astrocytes and the amplitude of the neuronal SIC is determined by the magnitude of the Ca²⁺ wave in astrocytes.

DISCUSSION

The existence of a bi-directional intercellular signaling pathway between neurons and astrocytes has been attested to by numerous recent reports (Dani et al., 1992; Charles et al., 1994; Porter and McCarthy, 1996; Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Pasti et al., 1998; Bezzi et al., 1998; Newman and Zahs 1998; Araque et al. 1998a,b). Ca²⁺ imaging studies have indicated that Ca²⁺ elevations in astrocytes, stimulate release of glutamate from these cells (Parpura et al., 1994) and lead to glutamate-dependent increases in the neuronal Ca²⁺ levels (Parpura et al., 1994; Hassinger et al., 1995; Pasti *et al.*, 1998; Bezzi *et al.*, 1998). We investigated the physiological

consequences of this glutamate-dependent astrocyte-induced neuronal Ca^{2+} elevations by evaluating its effects on the neuronal physiology. However, the experimental approach of applying glutamate to astrocytes to stimulate them to release glutamate would not have allowed us to assess the neuronal responses specifically to the glutamate released from astrocytes alone. To circumvent this problem, we stimulated astrocytes to release glutamate by application of the neuroligand PGE_2 , since it has been implicated in the glutamate-dependent astrocyte-neuron signaling (Bezzi et al., 1998). Recently, Bezzi et al. presented evidence that activation of glutamate receptors in astrocytes leads to further release of glutamate from these cells through a prostaglandin-mediated process. Direct application of the prostaglandin PGE_2 to acute brain slices of the hippocampal CA1 region, led to Ca^{2+} elevations in the astrocytes and consequent glutamate-mediated Ca^{2+} elevations in neurons. Using application of PGE_2 as the ligand-induced stimulus for astrocytes to release glutamate, we examined the effects of such astrocyte stimulation on neuronal physiology. We found that application of the neuroligand PGE_2 evokes a Ca^{2+} wave among astrocytes which then evokes two distinct neuronal responses; 1) a NMDA and non-NMDA receptor-dependent SIC and 2) an increase in the frequency of the miniature synaptic currents. Our results are in agreement with the recent reports of Araque et al. (1998a,b) that mechanical and electrical stimulation of astrocytes raises their internal Ca^{2+} levels and evokes glutamate-dependent currents in adjacent neurons, and further validate them in the physiological context by using application of an endogenously existing neuroligand as the stimulus to astrocytes.

One concern with the focal application of PGE_2 was the selectivity of the stimulus, and that the neuronal response may, at least in part, be a direct consequence of the application of the ligand. To determine whether the neuronal responses are mediated by the PGE_2 -induced Ca^{2+} elevations in astrocytes, we disrupted the propagation of Ca^{2+} waves by incubation with thapsigargin and by microinjection of the Ca^{2+} chelator, BAPTA, into astrocytes. Both experimental manipulations succeeded in preventing the Ca^{2+} elevations in astrocytes and blocked the appearance of a SIC in neurons. Although prevention of SIC upon incubation with thapsigargin does not rule out the possibility of thapsigargin acting

through the neuronal Ca^{2+} stores, taken together with the observation that microinjection of BAPTA specifically into astrocytes abolishes the neuronal responses, it clearly shows that the Ca^{2+} elevation in astrocytes is responsible for evoking the neuronal response.

Besides demonstrating the requirement of Ca^{2+} increase in astrocytes for evoking the neuronal SIC, our results further emphasize the Ca^{2+} dependence of the astrocyte-neuron signaling by providing a clear correlation between the strength of the astrocyte Ca^{2+} wave and the neuronal SIC. We used two distinct stimuli, mechanical stimulation and application of PGE_2 to astrocytes, and evaluated their abilities to evoke a Ca^{2+} wave among astrocytes and a SIC in neurons. Our study showed that the percentage of astrocytes that responded to these stimuli by initiating Ca^{2+} waves differed significantly, as did the percentage of astrocytes that evoked a neuronal SIC. Further analysis of the Ca^{2+} waves revealed that the magnitude of the Ca^{2+} response of astrocytes, evoked by the two stimuli was significantly different. The mean radius of the Ca^{2+} waves evoked by the PGE_2 application was significantly smaller than that evoked by mechanical stimulation ($P < 0.001$, Figure 5C). Correspondingly, the amplitudes of the neuronal SICs evoked by the two responses also varied significantly. The PGE_2 -evoked neuronal SICs were significantly smaller in amplitude than those evoked by the mechanical stimulation ($P < 0.001$, Figure 5D). Since the neuronal SIC is evoked by the Ca^{2+} -dependent glutamate release from astrocytes, it is conceivable that a larger Ca^{2+} wave involving a larger number of astrocytes, would lead to an overall higher amount of glutamate release and therefore, evoke a bigger SIC in neurons. Our results indicate that not only is a Ca^{2+} wave in astrocytes necessary for evoking a neuronal SIC, but that the neuronal response is graded and the amplitude of the SIC is determined by the magnitude of the Ca^{2+} wave.

With all the existing evidence, a scenario can be conceived in which synaptically released glutamate from neurons, through activation of glutamate receptors in astrocytes, could lead to a Ca^{2+} -dependent release of PGE_2 from astrocytes which could then modulate neuronal currents and excitability. What is the possible physiological significance of the PGE_2 -induced astrocyte-neuron signaling? PGE_2 has been shown to have profound effects in various physiological functions such as temperature regulation, circulation and pain

sensation (Feurstein et al., 1982; Milton, 1982; Halushka, 1989; Smith, 1989). Some of these effects could occur from its action in the central nervous system. Current data suggests that prostaglandins can also be directly involved in the nervous system pathology (Baran *et al.*, 1987; Malmberg and Yaksh, 1992; Collaco-Moraes *et al.*, 1996). In an intriguing example, release of the luteinizing hormone-releasing hormone (LHRH) involved in the sexual development, from the hypothalamic neurons appears to be mediated by release of PGE₂ from astrocytes (Rettori, 1992; Canteros 1995; Molina-Holgado, 1995; Mollace 1995; Martini, 1997). The proposed mechanism is that norepinephrine-induced release of nitric oxide (NO) from the NO-ergic neurons induces PGE₂ release from astrocytes by activation of the enzyme cyclooxygenase. Since, NO is an important putative second messenger in the nervous system implicated in a number of signal transduction pathways (Schuman and Madison, 1991; Haley et al, 1992), it further underscores the importance of the PGE₂-induced astrocyte-neuron signaling.

In summary, our results provide electrophysiological evidence for ligand-induced astrocyte-neuron signaling confirming our hypothesis that stimulation of an astrocyte by application of the neuroligand PGE₂ evokes a NMDA and non-NMDA glutamate receptor-dependent SIC in adjacent neurons in an astrocyte Ca²⁺-dependent manner and support a view of the CNS in which astrocytes and neurons participate in bidirectional communication.

REFERENCES

- Araque A, Parpura V, Sanzgiri RP, Haydon PG (1998a) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur J Neurosci* 10:2129-2142.
- Araque A, Sanzgiri RP, Parpura V, Haydon PG (1998b) Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J Neurosci* (in press).
- Barres BA (1991) New roles for glia. *J Neurosci* 11:3685-3694.

- Basarsky TA, Papura V, Haydon PG (1994) Hippocampal synaptogenesis in cell culture: developmental time course of synapse formation, calcium influx, and synaptic protein distribution. *J Neurosci* 14:6402-6411.
- Baran H, Heldt R, Hertting G (1987) Increased prostaglandin formation in rat brain following systemic application of kainic acid. *Brain Res* 404:107-112.
- Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Lodi Rizzini B, Pozzan T, Volterra A (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* 391:281-285.
- Canteras G, Rettori V, Franchi A, Genaro A, Cerbal E, Faletti A, Gimeno M, McCann SM (1995) Ethanol inhibits luteinizing hormone-releasing hormone (LHRH) by blocking the response of LHRH neuronal terminals to nitric oxide. *Proc Natl Acad Sci USA* 92:3416-3420.
- Charles AC (1994) Glia-neuron intercellular calcium signaling. *Dev Neurosci* 16:196-206.
- Charles AC, Dirksen ER, Merrill JE, Sanderson MJ (1993) Mechanisms of intercellular calcium signaling in glial cells studied with dantrolene and thapsigargin. *Glia* 7:134-145.
- Charles AC, Merrill JE, Dirksen ER, Sanderson MJ (1991) Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* 6:983-992.
- Collaco-Moraes Y, Asprey B, Harrison M, de Belleruche J (1996) Cyclooxygenase-2 messenger RNA induction in focal cerebral ischemia. *J Cereb Blood Flow Metab* 16:1366-1372.
- Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signalling. *Science* 247:470-473.
- Dani JW, Chernjavsky A, Smith SJ (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* 8:429-440.
- Feurstein G, Abelberg SA, Kopin IJ, Jacobowitz JD (1982) Hypothalamic sites for cardiovascular and sympathetic modulation by prostaglandin E₂. *Brain Res* 231:335-342.
- Finkbeiner SM (1992) Calcium waves in astrocytes-filling in the gaps. *Neuron* 8:1101-1108.

- Haley JE, Wilcox GL, Chapman PF (1992) The role of nitric oxide in hippocampal long-term potentiation. *Neuron* 8:211-216.
- Halushka P (1989) Thromboxane, prostaglandin and leukotriene receptors. *Annu Rev Pharmacol Toxicol* 29:213-239.
- Hassinger TD, Atkinson PB, Strecker GJ, Whalen LR, Dudek FE, Koseel AH, Kater SB (1995) Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. *J. Neurobiol.* 28:159-170.
- Malmberg AB, Yaksh TL (1992) Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. *Science* 257:1276-1279.
- Martini L, Motta M, Piva F, Zanisi M (1997) LHRF, LHRH, GnRH: what controls the secretion of these hormones? *Mol Psychiatry* 2:373-6.
- Milton AS (1982) Prostaglandins in fever and the mode of action of antipyretic drugs. *Pyretics and antipyretics*. Springer-Verlag, Berlin: 259-267.
- Molina-Holgado F, Lledo A, Gauza C (1995) Evidence for cyclooxygenase activation by nitric oxide in astrocytes. *Glia* 15:167-72.
- Mollace V, Colasanti M, Rodino P, Lauro GM, Rotiroti D, Nistico G (1995) NMDA-dependent prostaglandin E₂ release by human cultured astroglial cells is driven by nitric oxide. *Biochem Biophys Res Commun* 215:793-799.
- Nedergaard M (1994) Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* 263:1768-1771.
- Newman EA, Zahs KR (1998) Modulation of neuronal activity by glial cells in the retina. *J Neurosci* 18:4022-4028.
- Parpura V, Basarsky TA, Haydon PG (1996) Astrocyte-neuron signalling. In *Excitatory amino acids and the cerebral cortex*. Edited by F Conti, TP Hicks. MIT Press/Bradford Books, Cambridge. pp 167-174.
- Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte-neuron signalling. *Nature* 369:744-747.

- Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bi-directional form of communication between neurons and astrocytes *in situ*. *J Neurosci* 17:7817-7830.
- Porter JT, McCarthy KD (1996) Hippocampal astrocytes *in situ* respond to glutamate released from synaptic terminals. *J Neurosci* 16:5073-5081.
- Ransom BR, Orkand RK (1996) Glial-neuronal interactions in non-synaptic areas of the brain: studies in the optic nerve. *Trends Neurosci* 19:352-358.
- Rettori V, Gimeno M, Lyson K, McCann SM (1992) Nitric oxide mediates norepinephrine-induced prostaglandin E_2 release from the hypothalamus. *Proc Natl Acad Sci USA* 89:11543-11546.
- Schuman EM, Madison DV (1991) A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* 254:1503-1506.
- Smith WL (1989) The eicosanoids and their biochemical mechanisms of action. *Biochem J* 259 315-324.
- Smith SJ (1994) Neuromodulatory astrocytes. *Current Biology* 4:807-810.
- Theodosius DT, MacVicar B (1996) Neurone-glia interactions in the hypothalamus and pituitary. *Trends Neurosci* 19:363-367.
- Verkhratsky A, Kettenmann H (1996) Calcium signalling in glial cells. *Trends Neurosci* 19:346-352.

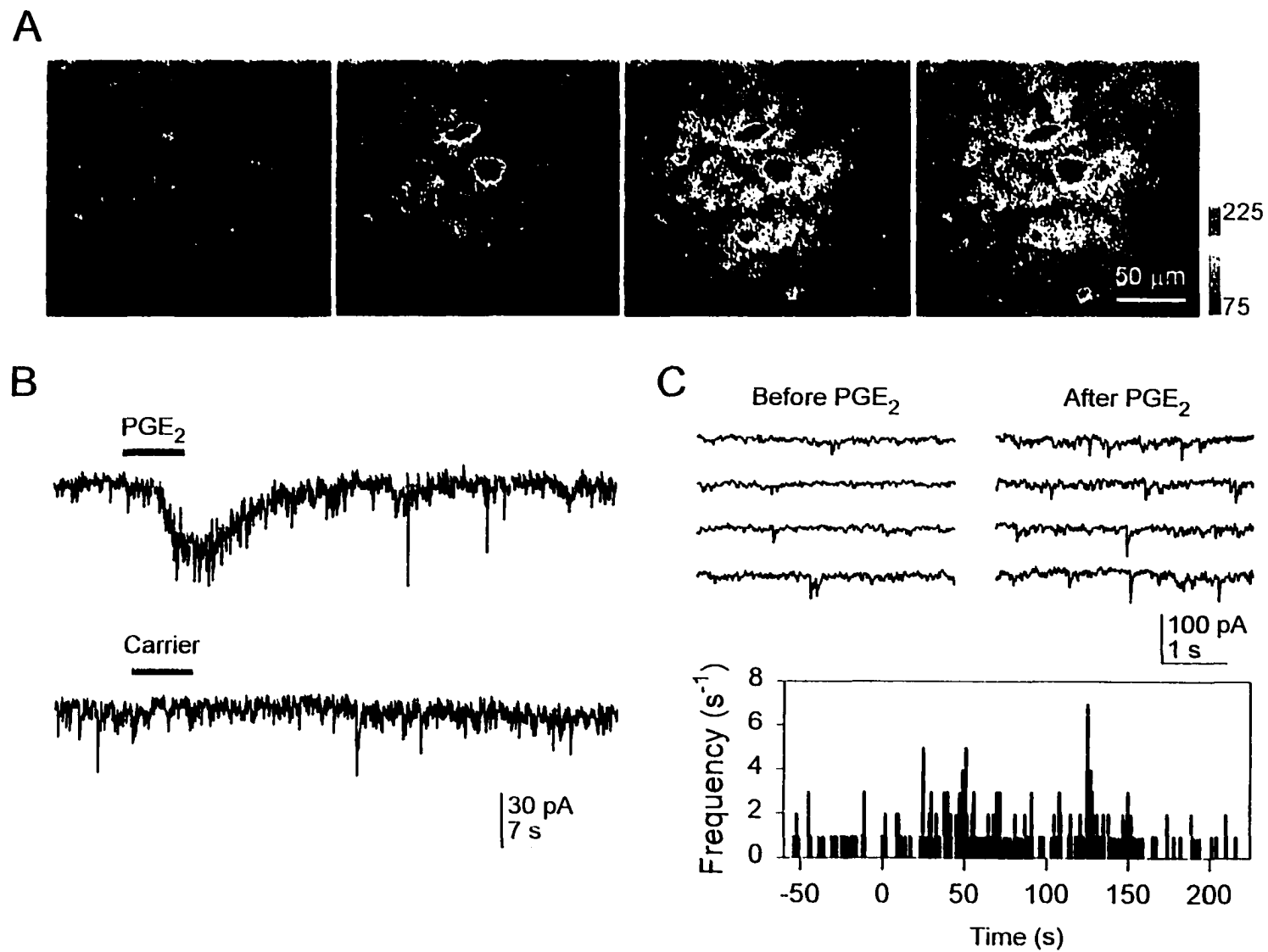


Figure 1

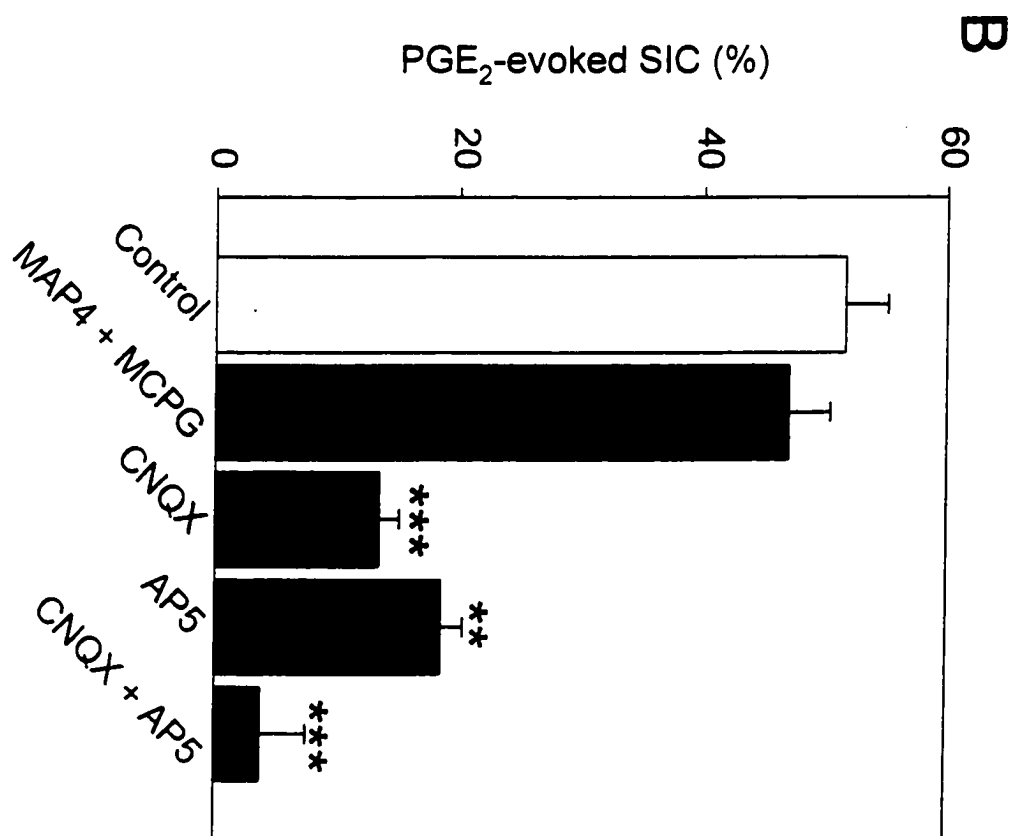
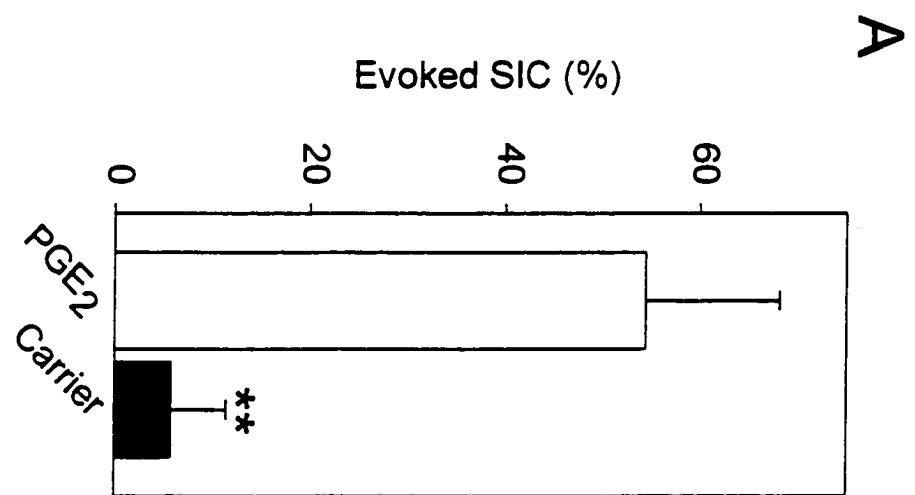


Figure 2

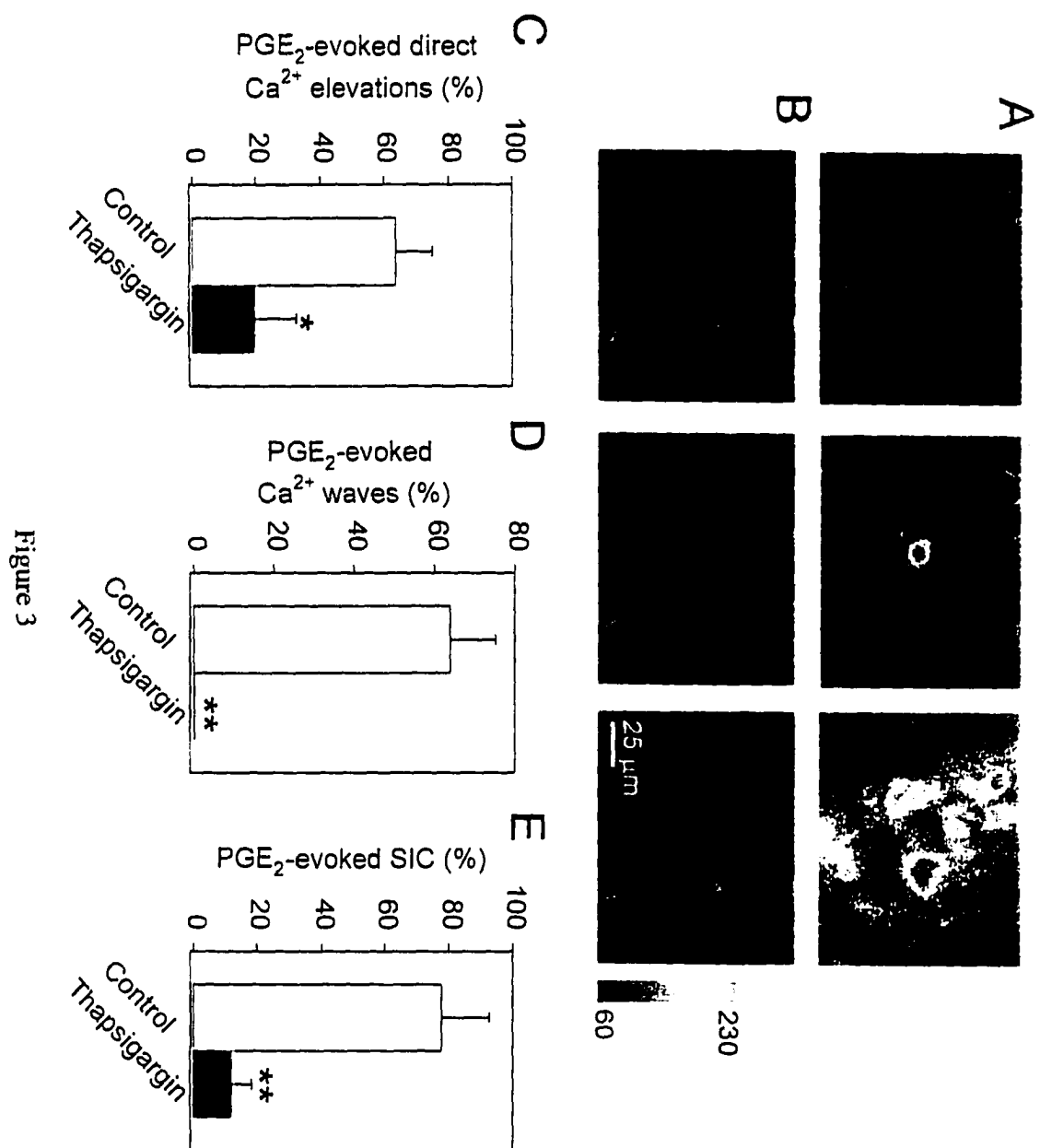
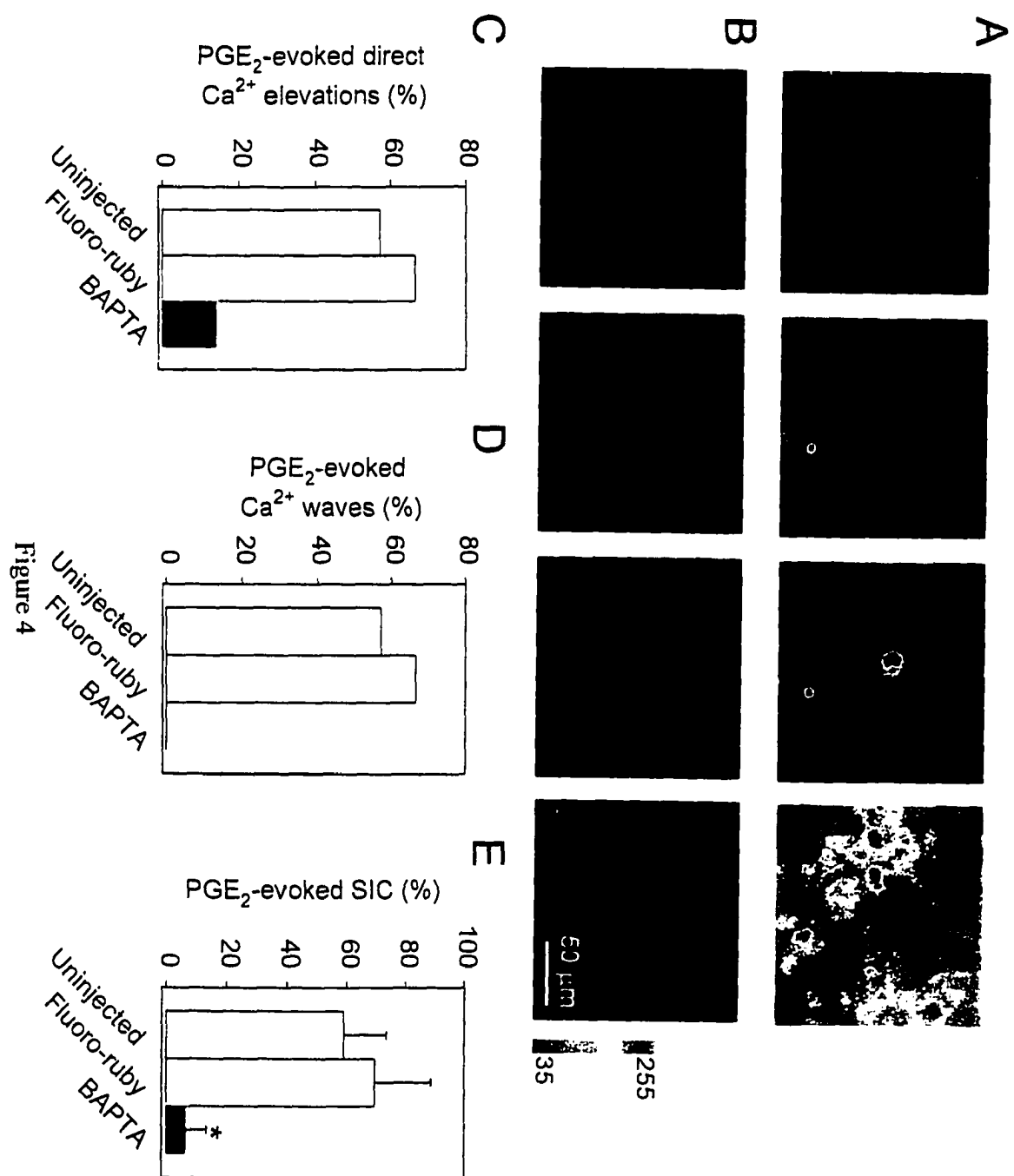


Figure 3



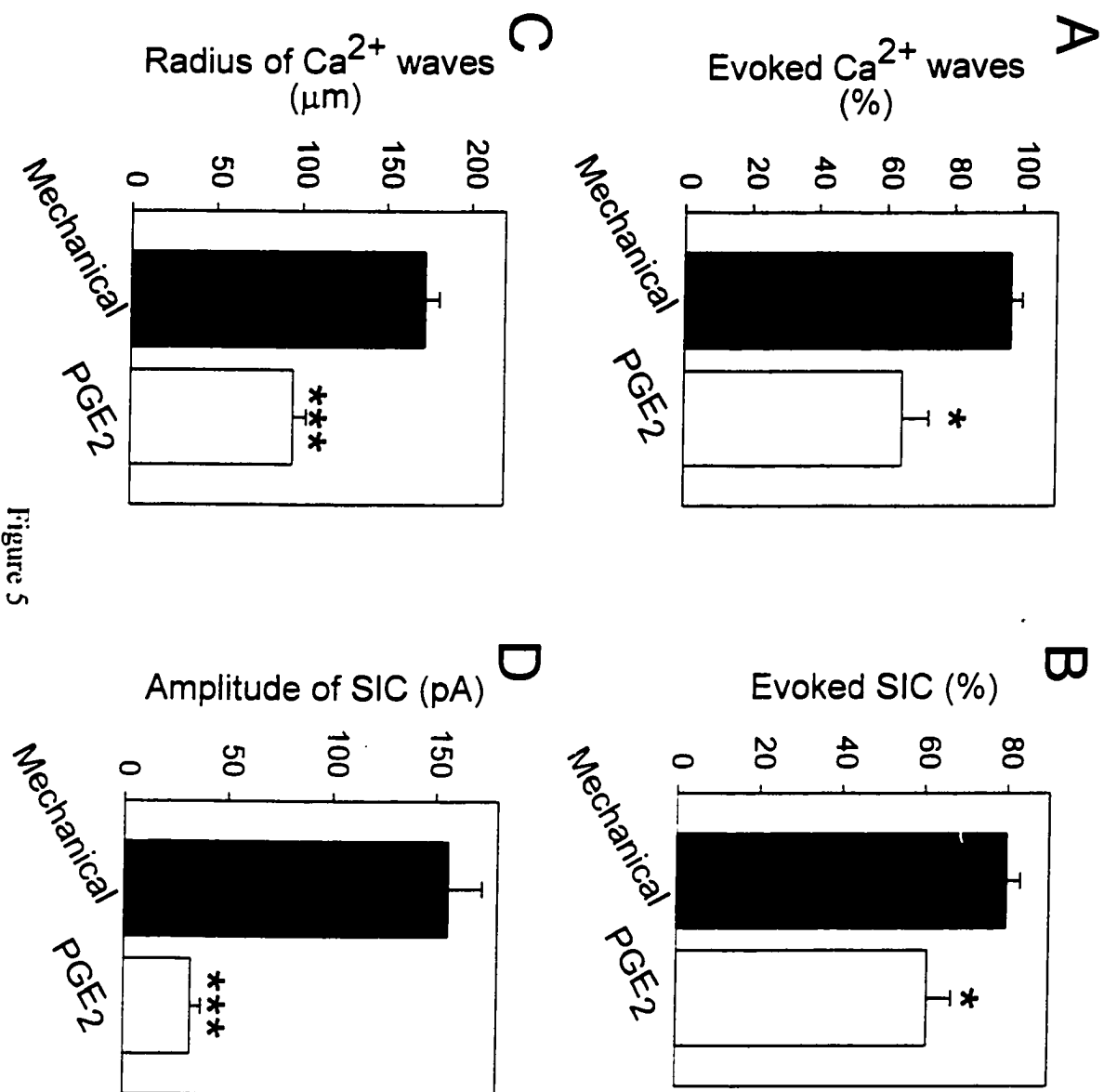


Figure 5

FIGURE LEGENDS

FIGURE 1. Effects of PGE₂ application. *A*, Application of PGE₂ to a mixed culture of neurons and astrocytes caused a Ca²⁺ elevation in the astrocyte which spread as a Ca²⁺ wave in adjacent astrocytes. *B* and *C* show the typical neuronal responses to PGE₂ application. *B*, A long-lasting, slow inward current (SIC) was evoked in adjacent neurons. Application of the carrier solution alone (1% ethanol) did not evoke a SIC. *C*, An increase of miniature postsynaptic currents (mPSCs) was seen in adjacent neurons. Top panel shows mPSCs recorded before and after PGE₂ application. Bottom panel shows the time course of the mPSC frequency calculated in 1 s bins. 0 time corresponds to the onset of PGE₂ application which lasted for 5 s.

FIGURE 2. PGE₂-evoked slow inward current is mediated by both NMDA and non-NMDA ionotropic glutamate receptors, but not by metabotropic glutamate receptors. *A*, Neuronal SIC is due to PGE₂ application, since application of the carrier solution did not evoke SIC. *B*, Percentage of PGE₂-evoked SIC in 0.5 mM MAP4, 0.5 mM MCPG, 20 μM CNQX, 50 μM D-AP5 and in the control solution in parallel cultures. *n* ≥ 3 for each group. Significant differences with respect to control were established by the Student's t-test at *p* < 0.05 (*), *p* < 0.01 (**) and *p* < 0.001 (***).

FIGURE 3. Thapsigargin prevents spreading of calcium elevations among astrocytes and reduces the frequency of astrocyte-induced neuronal SIC. *A*, In a control experiment, application of PGE₂ caused Ca²⁺ elevation in the stimulated cell followed by calcium elevations in adjacent astrocytes. In contrast, in *B*, treatment of a sister culture with 1 μM thapsigargin prevented the spread of calcium elevations in astrocytes. PGE₂ was applied between the left and middle images. Images were acquired every 2 seconds. Pseudocolor representation indicates intracellular calcium levels, where purple/blue represent basal calcium levels while yellow/red increased calcium levels. Quantitation of calcium imaging studies show that thapsigargin significantly reduced the ability of PGE₂ to induce Ca²⁺ elevations in astrocytes (*C*), and also significantly reduced the spread of the calcium wave

to adjacent astrocytes (*D*). Cells were identified as participating in the Ca^{2+} wave if the fluo-3 signal changed $\geq 30\%$ of the baseline. *E*. In parallel electrophysiological experiments treatment of cultures with thapsigargin reduced the frequency with which PGE_2 application evoked a neuronal SIC. Numbers of repetitions are ≥ 6 in all categories. Significant differences with respect to control were established by the Student's t-test at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

FIGURE 4. Microinjection of the calcium chelator BAPTA into an astrocyte prevents astrocyte calcium waves and blocks astrocyte-induced neuronal signaling. In *A* and *B*, cells were loaded with the calcium indicator fluo-3 to monitor the ability of PGE_2 application to induce calcium elevations in astrocytes. In *A*, a control experiment is shown in which a single astrocyte was microinjected with a carrier solution containing fluoro-ruby (left panel). The next three panels show images in pseudocolor mode representing intensity of fluo-3 emission. PGE_2 was applied after the first panel. PGE_2 increases intracellular calcium in the injected cell as well as in neighboring unstimulated astrocytes. In contrast, *B* shows the result of injecting fluoro-ruby and BAPTA into one astrocyte (left panel). PGE_2 -stimulation of the injected cell did not change the fluorescent emission of fluo-3 either in the stimulated cell or the neighboring astrocytes (compare the central and right panels taken before and after mechanical stimulation). *C* and *D* show quantitative data taken from these experiments. BAPTA reduces the ability of PGE_2 to elevate calcium levels in the directly stimulated cell (*C*) and the incidence of calcium wave. In parallel studies neuronal currents were recorded in response to mechanical stimulation of astrocytes. While injection of fluoro-ruby did not prevent an astrocyte-induced SIC (*E*), injection of BAPTA prevented the appearance of a SIC following PGE_2 application (*E*, $p < 0.01$). (**) represents $p < 0.01$, *** represents $p < 0.001$). In all categories $n \geq 6$.

Figure 5. Occurrence and amplitude of the neuronal SIC depends upon the Ca^{2+} wave in astrocytes. *A*, Percentage of Ca^{2+} waves evoked by PGE_2 is significantly smaller than when evoked by mechanical stimulation ($p < 0.05$). *B*, Similarly, percentage of the astrocyte-

induced neuronal SIC evoked by PGE_2 is smaller than when evoked by mechanical stimulation ($p < 0.05$). *C*, Comparison between the waves evoked by PGE_2 application and mechanical stimulation. *C*, Radius of the Ca^{2+} wave evoked by PGE_2 application is significantly smaller than that caused by mechanical stimulation ($p < 0.001$). *D*, Correspondingly, amplitude of the PGE_2 -evoked SIC is smaller than the SIC evoked by mechanical stimulation ($p < 0.001$).

CHAPTER 5. GENERAL CONCLUSIONS

General discussion

During the last decade our understanding of the glial cells and their roles in the CNS has changed considerably. It is becoming increasingly clear that glial cells may play more dynamic signaling roles than previously thought. This change in perception can be attributed to three main findings;

- 1) Astrocytes, a subtype of glial cells, exhibit a novel form of excitability and mode of communication based on variations in their Ca^{2+} concentrations (Cornell-Bell et al., 1990; Charles et al., 1991; Finkbeiner, 1992; Newman and Zahs, 1997).
- 2) Neuronal activity can trigger these Ca^{2+} waves among astrocytes (Dani et al. 1992; Porter and MacCarthy, 1996).
- 3) Elevations in the intracellular Ca^{2+} concentration in astrocytes can evoke elevations in the Ca^{2+} levels of adjacent neurons (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995; Pasti et al., 1997; Bezzi et al., 1998).

One of the most intriguing questions arising after the demonstration of the existence of astrocyte-to-neuron signaling concerns the physiological consequences of such communication on the modulation of neuronal activity and/or synaptic transmission. The studies performed in this thesis demonstrate that astrocyte-to-neuron signaling may indeed modulate neuronal activity and synaptic transmission (Araque et al., 1998a,b, Chapter 2, 3, 4) in cultured hippocampal cells. Stimuli that increase the intracellular Ca^{2+} in astrocytes, generate a glutamate-dependent slow inward current (SIC) which is mediated by the NMDA and non-NMDA glutamate receptors and/or an increase in the frequency of the miniature synaptic currents in adjacent neurons mediated by the NMDA receptors.

A variety of stimuli that raise astrocyte Ca^{2+} can evoke astrocyte-to-neuron signaling

A variety of stimuli (mechanical, electrical and application of PGE_2) were used to elicit

astrocyte-to-neuron signaling. All the three stimuli successfully caused Ca^{2+} elevations in astrocytes which were found to be necessary and sufficient for evoking neuronal responses.

Mechanical stimulation: Both, mechanical and electrical stimuli cause a Ca^{2+} elevation in the stimulated astrocyte which is followed by similar Ca^{2+} increases in the surrounding cells. The Ca^{2+} increase in the stimulated cell is thought to occur by an influx of extracellular Ca^{2+} , since in the absence of extracellular Ca^{2+} there is little change in the Ca^{2+} concentration (Charles et al., 1991). The mechanism by which the mechanical stimulus is transduced into a Ca^{2+} influx is not understood completely. One possibility is that Ca^{2+} influx occurs through stretch-activated ion channels, although a transient puncture of cell membrane cannot be ruled out (Charles et al., 1991). Stretch-activated channels have been demonstrated in astrocytes and a number of other cell types (Sachs, 1988). The regenerative, non-decremental spread of the Ca^{2+} wave through the network of astrocytes has been proposed to involve two mechanisms, diffusion of the second messenger IP_3 which causes Ca^{2+} release from the IP_3 -sensitive intracellular Ca^{2+} stores and the Ca^{2+} -dependent- Ca^{2+} -release from the internal stores (Charles, 1991; Wang et al., 1997). The Ca^{2+} waves observed during this study, are mediated by an IP_3 -mediated mechanism since in the presence of thapsigargin, (a Ca^{2+} -ATPase inhibitor that depletes the Ca^{2+} internal stores and prevents the spread of astrocyte Ca^{2+} waves), the spread of Ca^{2+} waves in astrocytes (Chapter 2, figure 3; Chapter 3) was suppressed. However, an additional mechanism may exist, since sporadic Ca^{2+} waves were still observed after incubation with thapsigargin. Since, the mechanically induced initial Ca^{2+} elevation in the stimulated astrocyte, was not blocked by thapsigargin, it suggests that the Ca^{2+} elevation itself, does not require release from the IP_3 -sensitive Ca^{2+} stores, consistent with the idea of Charles et al. (1991), that it occurs via an influx of Ca^{2+} .

How an electrical stimulation causes Ca^{2+} elevations in astrocytes is still unknown. It was suggested that since astrocytes express voltage gated Ca^{2+} channels, these may contribute to the Ca^{2+} influx (MacVicar, 1984; MacVicar et al., 1991; Kettenmann 1996). However a recent report indicates that these channels do not contribute to the Ca^{2+} increase occurring in astrocytes upon depolarization (Carmignoto et al., 1998).

PGE₂-evoked Ca²⁺ waves: Recently, prostaglandins, predominantly prostaglandin E₂ (PGE₂), have been shown to evoke Ca²⁺ elevations in astrocytes and stimulate Ca²⁺-dependent glutamate release in acute brain slices (Bezzi *et al.*, 1998). I found that focal application of PGE₂ reliably evokes Ca²⁺ waves in astrocytes (Chapter 4). The mechanism by which PGE₂ directly increases Ca²⁺ and the steps that link Ca²⁺ rise to glutamate release are still unclear. However, thapsigargin blocked the PGE₂-evoked direct Ca²⁺ elevations and propagation of the Ca²⁺ waves, indicating that a release from IP₃-sensitive internal stores occurs during the initiation as well as propagation of the PGE₂-evoked Ca²⁺ waves (Chapter 4, figure 3). Bezzi *et al.* (1998) reported that the PGE₂-evoked Ca²⁺ elevations were partially blocked by antagonists of the AMPA receptors and the metabotropic glutamate receptors, suggesting that these Ca²⁺ elevations are in part mediated through these receptors on astrocytes, which are activated by the glutamate released by astrocytes in response to the PGE₂ application.

A concern with the focal application of PGE₂ was the specificity of the stimulus, and whether the PGE₂ application was restricted to only one astrocyte. However, PGE₂ application to an astrocyte previously injected with the Ca²⁺ chelator BAPTA did not evoke a Ca²⁺ wave (Chapter 4, figure 4) demonstrating that the diffusion of PGE₂ from the puff pipette onto neighboring astrocytes was not significant enough to evoke responses from them.

Ca²⁺ elevation in astrocytes is necessary and sufficient to induce astrocyte-neuron signaling

The Ca²⁺ dependence of glutamate release from astrocytes has been demonstrated with several experimental approaches. For example, thapsigargin, strongly reduced the incidence of the astrocyte-induced SIC (Chapter 2, figure 3; Chapter 3; Chapter 4, figure 3) as well as the increase in mPSCs in neurons (Chapter 3). Additionally, BAPTA-AM (the cell permeant acetoxymethyl derivative of the Ca²⁺ chelator BAPTA), also suppressed the astrocyte-to-neuron signaling. These data indicate that intracellular Ca²⁺ elevations in astrocytes are required to stimulate glutamate release and to evoke astrocyte-to-neuron

signaling. However, the possibility that thapsigargin and BAPTA-AM mediate their action by directly affecting the neuronal Ca^{2+} cannot be discounted.

To address this issue, that is to test the astrocyte Ca^{2+} -dependence of the astrocyte-neuron signaling, I developed the following approach. Ca^{2+} elevations in specific astrocytes were blocked by micro-injecting the Ca^{2+} chelator BAPTA into specific astrocytes and then such injected astrocytes were stimulated to evoke neuronal responses. To identify the injected cell, the fluorescent dye, fluororuby was added to the injection solution. Fluororuby contains a dextran moiety which prevents its passage through gap junctions, restricting the dye to the injected cell. After a recovery period, injected cells (identified by the red fluorescence of fluororuby) were stimulated. Stimulation of a BAPTA-injected astrocyte, either mechanically or by PGE_2 -application neither induced Ca^{2+} elevations in them, nor elicited neuronal responses. This experiment conclusively proves that a Ca^{2+} elevation within astrocytes is necessary to evoke astrocyte-neuron signaling. It also controls for the possibility that the neuronal responses are evoked by a direct action of PGE_2 on neurons, instead of being mediated through astrocytes.

In another set of experiments, I injected the Ca^{2+} -cage NP-EGTA along with fluororuby into astrocytes. Elevation of astrocyte Ca^{2+} by photolysis of NP-EGTA, evoked a SIC and increase in the mPSCs in adjacent neurons, indicating that a Ca^{2+} elevation in astrocytes is sufficient to evoke astrocyte-to-neuron signaling (Chapter 3, figure 4).

Taken together these experiments demonstrate that an astrocyte Ca^{2+} elevation is necessary and sufficient to evoke astrocyte-neuron signaling.

Besides demonstrating the requirement for Ca^{2+} increase in astrocytes for evoking the neuronal SIC, my results further emphasize the Ca^{2+} dependence of the astrocyte-neuron signaling by showing that the neuronal response to the astrocyte Ca^{2+} wave is graded and depends on the strength of the astrocyte Ca^{2+} wave. Ca^{2+} waves evoked by PGE_2 application propagated over a smaller distance and involved fewer cells than those evoked by a mechanical stimulation (mean radius, mechanically-evoked Ca^{2+} wave = 172.68 ± 8.54 μm , $n = 10$; PGE_2 -evoked Ca^{2+} wave = 94.95 ± 7.81 μm , $n = 19$) (Chapter 4, figure 5). Corresponding to these differences in the Ca^{2+} waves, the amplitude of the PGE_2 -evoked

SIC is smaller than that of the mechanically evoked SIC (155.79 ± 16.63 pA with mechanical stimulation, $n = 63$; 32.01 ± 5.11 , $n = 44$ with focal application of PGE_2) (Chapter 4, figure 5). Since the neuronal SIC is evoked by the Ca^{2+} -dependent glutamate release from astrocytes, it is conceivable that a larger Ca^{2+} wave involving a larger number of astrocytes, would lead to an overall higher amount of glutamate release and therefore, evoke a bigger SIC in neurons.

The probable picture of astrocyte-neuron signaling that emerges out of the existing evidence, is as follows. Synaptically released glutamate released from neurons may bind to the AMPA receptors and the metabotropic glutamate receptors, in particular the mGluR_1 , on astrocytes (Bezzi et al., 1998). Activation of these receptors leads to a Ca^{2+} -dependent prostaglandin synthesis in astrocytes (Bezzi et al., 1998) through a G-protein mediated signal transduction cascade involving the breakdown of DAG by the enzyme PLA_2 into arachidonic acid (AA). An early event in this series is production of IP_3 and DAG by the enzyme PLC. IP_3 can mediate release of Ca^{2+} from the internal stores, and part of the Ca^{2+} elevations in astrocytes observed in response to glutamate may be due to this mechanism. AA is the substrate for enzyme cyclooxygenase to produce prostaglandins. Prostaglandins are produced and probably released by astrocytes to the extracellular space where they bind to their receptors on neighboring astrocytes as well as the releasing astrocytes and cause further Ca^{2+} elevations in these cells (Bezzi et al., 1998; Chapter 4) and a Ca^{2+} -dependent glutamate release from them (Bezzi et al., 1998; Chapter 4). The Ca^{2+} elevations in neurons were abolished by addition of glutamate receptor antagonists, indicating that PGE_2 does not evoke Ca^{2+} elevations in neurons directly (Bezzi et al., 1998).

Consistent with this, the Ca^{2+} -dependent glutamate release from astrocytes, can be mimicked by external application of PGE_2 to astrocytes. How PGE_2 evokes Ca^{2+} elevation and how this Ca^{2+} elevation in turn evokes glutamate release is not understood. It is clear though, that raising Ca^{2+} levels in astrocytes by various other stimuli such as, mechanical, electrical or photolysis of a Ca^{2+} cage (Parpura et al., 1994; Araque et al., 1998a; Chapter 2; Araque et al., 1998b; Chapter 3; Chapter 4) is sufficient to evoke glutamate release from astrocytes.

The glutamate released from astrocytes can bind to receptors on surrounding astrocytes and cause further Ca^{2+} elevations and PGE_2 release from them. Also, it can bind to glutamate receptors on adjacent neurons, and evoke neuronal Ca^{2+} elevations. As shown in this dissertation it can evoke two primary neuronal electrical responses, a neuronal SIC and/or an increase in the mPSCs.

Physiological consequences of the astrocyte-to-neuron signaling

Modulation of the neuronal electrical activity: As reported in Chapter 2, 3 and 4, the mechanically induced astrocyte-evoked SIC amplitude at around the resting membrane potential (~ -60 mV) was ~ 50 pA in control solution (Araque et al., 1998a,b). In current clamp conditions, this current evoked small long-lasting depolarizations that eventually can trigger a train of action potentials in previously silent neurons (cf. Hassinger et al., 1995; Araque et al., 1998a,b). In agreement with this result, it has been recently shown that astrocyte Ca^{2+} waves can modulate the light-evoked spike activity in retinal neurons probably through glutamate-mediated activation of inhibitory interneurons (Newman and Zahs, 1998).

Modulation of synaptic transmission: Another exciting possibility arising from the existence of the astrocyte-to-neuron signaling is whether astrocytes can modulate synaptic transmission. As mentioned above, astrocyte stimulation can induce an increase in the frequency of miniature excitatory and inhibitory postsynaptic currents, without changing their amplitudes. Since astrocyte stimulation can depolarize neurons beyond the firing threshold, at least in some cases, this increase in spontaneous PSC frequency is probably due to astrocyte-induced suprathreshold depolarizations of presynaptic neurons that elicited action potential-evoked PSCs.

However, alternative mechanisms are involved because this modulation of the synapse is observed even in the presence of tetrodotoxin (TTX, a toxin that blocks sodium channels preventing action potential firing) (Araque et al., 1998 a, 1998b, Chapter 2, Chapter 3; Chapter 4). Since the frequency of the mPSCs is increased while their amplitudes remain unchanged, it suggests that astrocytes may directly influence the state of the presynaptic

terminal increasing the probability of neurotransmitter release from nerve terminals (Araque et al., 1998a,b). This effect is mediated by activation of NMDA receptors on the presynaptic neurons.

In addition to this, astrocytes can directly modulate evoked synaptic transmission between cultured hippocampal neurons. Stimulation of astrocytes transiently reduces the amplitude of both action potential-evoked excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs, respectively), without modifying the mean amplitude of the miniature synaptic currents (Araque et al., 1998a; chapter 2; Araque et al., 1998b; chapter 3). This astrocyte-induced reduction of synaptic currents is sensitive to antagonists of mGluRs. Activation of metabotropic glutamate receptors is known to reduce excitatory and inhibitory transmitter release from presynaptic terminals (Forsythe and Clements, 1990; Baskys and Malenka, 1991; Gereau and Conn, 1995). Following the current views of the mechanism underlying the effects of mGluRs on synaptic transmission, activation of these receptors by glutamate released from astrocytes would modulate voltage gated Ca^{2+} channels through G-protein-mediated pathways, thus reducing the Ca^{2+} influx evoked by the action potential and subsequently the amount of transmitter released (Forsythe and Clements, 1990; Takahashi et al., 1996). Therefore, astrocytes may directly modulate synaptic transmission by releasing glutamate that acting on presynaptic mGluRs can reduce the probability of transmitter release from these presynaptic terminals (Araque et al., 1998a).

Thus, astrocyte-to-neuron signaling can be manifested at different levels in neurons. At the postsynaptic neuron, it can be revealed as slow inward currents or slow depolarizations mediated by the activation of NMDA and non-NMDA glutamate receptors. The astrocyte-induced slow depolarization can eventually be suprathreshold, then increasing the frequency of action potential-evoked synaptic transmission. In the presynaptic terminal, glutamate released from astrocytes can increase the probability of spontaneous transmitter release through an NMDA receptor-dependent mechanism. In addition, glutamate released from astrocytes may decrease the probability of action potential-evoked transmitter release through the activation of presynaptic mGluRs.

Conclusions and perspectives

Based on the results reviewed above, our current knowledge of communication between neurons and astrocytes can be summarized as follows. Neuronal activity evokes transmitter release from synaptic terminals that eventually can escape from the synaptic cleft, activating neurotransmitter receptors in adjacent astrocytes, and consequently evoking Ca^{2+} elevations in them. Interestingly, different levels of synaptic activity are able to evoke varying levels of astrocyte responses (Porter and McCarthy, 1996), suggesting that a different amount of neuronal activity would evoke a different amount of neurotransmitter spillover from the synaptic cleft, activating a different proportion of astrocyte receptors, and finally, evoking a different magnitude of astrocyte response.

The neuronal-induced, neurotransmitter-mediated Ca^{2+} elevations in astrocytes can then propagate to adjacent astrocytes forming nondecremental, regenerative Ca^{2+} waves that could spread slowly over relatively long distances, constituting a form of communication between astrocytes. These Ca^{2+} elevations in astrocytes stimulate the Ca^{2+} -dependent release of glutamate from astrocytes that can activate glutamate receptors in neurons to regulate neuronal Ca^{2+} levels, neuronal electrical activity and synaptic transmission. My studies indicate that neuronal response to astrocyte Ca^{2+} elevations is also graded (Chapter 4), since a bigger Ca^{2+} wave involving a greater number of astrocytes, evokes a larger neuronal SIC. This graded characteristic of neuron-to-astrocyte and astrocyte-to-neuron signaling may provide a spatial and temporal control of this neuromodulatory mechanism.

Synaptically released glutamate is one of the most firm candidates to mediate the neuron-to-astrocyte signaling, because neuronal-induced responses in astrocytes are strongly reduced by antagonists of metabotropic and AMPA glutamate receptors (Porter and McCarthy, 1996, Pasti et al., 1997; Araque et al., 1998a; 1998b; Bezzi et al., 1998). However, astrocytes *in situ* also express many receptors for other synaptically released neurotransmitters, such as adenosine, ATP or norepinephrine, that are coupled to intracellular pathways leading to increases in $[\text{Ca}^{2+}]_i$ in astrocytes (Cornell-Bell et al., 1990; Charles et al., 1991; Porter and McCarthy, 1996; Duffy and MacVicar, 1995; Salter and Hicks, 1995; Newman and Zahs,

1997). The possible contributions of other neurotransmitters to the neuron-to-astrocyte signaling have not yet been elucidated.

While the Ca^{2+} -dependence of the glutamate-mediated astrocyte-to-neuron signaling is firmly established, the signaling pathways linking ligand receptor interaction, Ca^{2+} elevation in astrocytes, and glutamate release are still poorly understood. Nevertheless, some important steps have been recently revealed by Bezzi et al. (1998), who proposed that coactivation of AMPA and metabotropic glutamate receptors in astrocytes stimulates phospholipase A_2 and Ca^{2+} -dependent arachidonic acid production, leading to the synthesis of prostaglandins that trigger Ca^{2+} -dependent events which result in glutamate release. However, a clear understanding of the signal transduction pathways involved is still required.

The studies presented in this dissertation were done using primary cultured hippocampal cells. Studies using cell cultures yield valuable insights into single cell physiology and behavior as well as mechanisms underlying intercellular signaling. However, it must be pointed out that the highly organized spatial structure of the CNS is not maintained in cell culture. For example, neurons in the hippocampus are organized into three major areas, the dentate gyrus, the CA3 region and the CA1 region. The axons of the neurons in the dentate gyrus, synapse on the pyramidal neurons of the CA3 region forming the mossy fiber pathway, and those of the pyramidal cells from the CA3 region onto the pyramidal cells in the CA1 region forming the schaffer collateral fiber pathway. This specific connectivity is lost in cell cultures. Since astrocytes in cell cultures, have diverse effects on neuronal physiology, (a SIC mediated through the NMDA and non-NMDA glutamate receptors, an increase in the frequency of mPSCs through the NMDA receptors, and a decrease in the amplitude of the evoked PSPs through the metabotropic glutamate receptors), it would be interesting to see whether these diverse neuromodulatory functions are specific to different parts of the intact hippocampus. Astrocyte-neuron signaling has already been demonstrated in hippocampal slice cultures as well as acutely dissociated hippocampal slices on the basis of Ca^{2+} imaging (Dani et al., 1992; Porter and MacCarthy, 1996; Pasti et al., 1997; Bezzi et al.,

1998). It will be important to study the electrophysiological aspects of the astrocyte-neuron signaling in hippocampal slices and finally in the intact brain.

Given that astrocytes far outnumber neurons in different regions of the CNS and given that astrocytes can divide and proliferate unlike the terminally differentiated neurons, the demonstration that astrocytes can participate directly in intercellular signaling assumes great significance in the normal physiology and pathology of the CNS. For instance, NMDA receptors have been implicated in long term potentiation (LTP) and memory (Madison et al., 1991; Malgaroli and Tsien, 1992; Manabe et al., 1992; Bliss and collingridge, 1993). Since glutamate released from astrocytes activates NMDA receptors, it is tempting to speculate that astrocytes may be involved in LTP and the formation of memory. In another example, astrocyte-neuron signaling might be involved in a CNS disorder, epilepsy. Epilepsy is a condition in the brain, in which seizures occur repetitively, unrelated to an acute infection or metabolic disorder. Seizures are caused by a synchronization of neuronal activity in a large population of central neurons. The bidirectional astrocyte-neuron signaling pathway which occurs through the intermediate step of an interastrocyte Ca^{2+} wave that can spread slowly over a long distance, could contribute to the epileptic disorder.

In conclusion, during the last few years considerable evidence has been accumulated supporting a more active role for astrocytes in the brain. Indeed, neurons can signal to astrocytes through synaptically released neurotransmitters, astrocytes communicate between themselves through Ca^{2+} waves, and astrocytes in turn can signal to neurons by releasing glutamate that can increase the neuronal Ca^{2+} levels, and can modulate neuronal electrical activity and synaptic transmission. Therefore, a new view of the CNS can be postulated in which astrocytes and neurons may function as a network where bi-directional communication takes place.

References

- Araque, A., Parpura, V., Sanzgiri, R.P., and Haydon, P.G. 1998a. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur. J. Neurosci.* **10**: 2129-2142.

- Araque, A., Sanzgiri, R.P., Parpura, V., and Haydon, P.G. 1998b. Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J. Neurosci.* (in press).
- Barde, Y.-A. 1989. Trophic factors and neuronal survival. *Neuron* **2**: 1525-1534.
- Baskys, A. and Malenka, R.C. (1991) Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. *J. Physiol. (Lond.)*, **444**: 687-701.
- Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Lodi Rizzini, B., Pozzan, T., and Volterra, A. 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* **391**: 281-285.
- Carmignoto, G., Pasti, L., and Pozzan, T. 1998. On the role of voltage-dependent calcium channels in calcium signaling of astrocytes *in situ* *J Neurosci* **18**: 4637-4645
- Charles, A.C. 1994. Glia-neuron intercellular calcium signaling. *Dev. Neurosci.* **16**: 196-206.
- Charles, A.C., Merrill, J.E., Dirksen, E.R., and Sanderson, M.J. 1991. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* **6**: 983-992.
- Choi, D.W. 1988. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**: 623-634.
- Coles, J.A. 1995. In *Neuroglia* pp. 793-804. Oxford University Press.
- Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., and Smith, S.J. 1990. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* **247**: 470-473.
- Dani, J.W., Chernjavsky, A., and Smith, S.J. 1992. Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* **8**: 429-440.
- Duffy, S., and MacVicar, B.A. 1995. Adrenergic calcium signaling in astrocyte networks within the hippocampal slice. *J. Neurosci.* **15**: 5535-5550.

- Engle, J., Schubert, D. and Bohn M.C. 1991. Conditioned media derived from glial cell lines promote survival and differentiation of dopaminergic neurons in vitro: Role of mesencephalic glia. *J Neurosci. Res.* **30**: 359-371.
- Finkbeiner, S.M. 1992. Calcium waves in astrocytes - Filling in the gaps. *Neuron* **8**: 1101-1108.
- Forsythe, I.D., and Clements, J.D. 1990. Presynaptic glutamate receptors depress excitatory monosynaptic transmission between mouse hippocampal neurons. *J. Physiol. Lond.* **429**: 1-16.
- Gaul, G. and Lubbert, H. 1992. Cortical astrocytes activated by basic fibroblast growth factor secrete molecules that stimulate differentiation of mesencephalic dopaminergic neurons. *Proc. R. Soc. Lond. B.* **249**: 57-63.
- Gereau, R.W. and Conn, P.J. (1995) Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. *J. Neurosci.*, **15**, 6879-6889.
- Hassinger, T.D., Atkinson, P.B., Strecker, G.J., Whalen, L.R., Dudek, F.E., Koseel, A.H., and Kater, S.B. 1995. Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. *J. Neurobiol.* **28**: 159-170.
- Kettenmann, H. 1996. Beyond the neuronal circuitry. *Trends Neurosci.* **19**: 305-306.
- Lin, L.-F., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993) GDNF: A glia cell derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**: 1130-1132.
- MacVicar, B.A. (1984) Voltage-dependent calcium channels in glial cells. *Science* **226**: 1345-1347.
- MacVicar, B.A., Hochman, D., Dealy, M.J., and Weiss, S (1991) Modulation of intracellular Ca^{2+} in cultured astrocytes by influx through voltage-activated Ca^{2+} channels. *Glia* **4**: 448-455.
- Nedergaard, M. 1994. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* **263**: 1768-1771.

- Newman, E.A., and Zahs, K.R. 1997. Calcium waves in retinal glial cells. *Science* **275**: 844-847.
- Newman, E.A., and Zahs, K.R. 1998. Modulation of neuronal activity by glial cells in the retina. *J. Neurosci.* **18**: 4022-4028.
- Parpura, V., Basarsky, T.A., Liu, F., Jęftinija, K., Jęftinija, S., and Haydon, P.G. 1994. Glutamate-mediated astrocyte-neuron signalling. *Nature* **369**: 744-747.
- Pasti, L., Volterra, A., Pozzan, T., and Carmignoto, G. 1997. Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes *in situ*. *J. Neurosci.* **17**: 5073-5081.
- Porter, J.T., and McCarthy, K.D. 1996. Hippocampal astrocytes *in situ* respond to glutamate released from synaptic terminals. *J. Neurosci.* **16**: 5073-5081.
- Sachs, F. 1988. Mechanical transduction in biological systems. *CRC Crit. Rev. Biol. Eng.* **16**: 141-169.
- Salter, M.W., and Hicks, J.L. 1995. ATP causes release of intracellular Ca^{2+} via the phospholipase C / IP_3 pathway in astrocytes from the dorsal spinal cord. *J. Neurosci.* **15**: 2961-2971.
- Takahashi, T., Forsythe, I.D., Tsujimoto, T., Barnes-Davies, M., and Onodera, K. 1996. Presynaptic calcium current modulation by a metabotropic glutamate receptor. *Science* **274**: 594-597.
- Venance, L., Stella, N., Glowinski, J., and Giaume, C. 1997. Mechanism involved in initiation and propagation of receptor-induced intercellular calcium signaling in cultured rat astrocytes. *J. Neurosci.* **17**: 1981-1992.
- Verkhratsky, A., and Kettenmann, H. 1996. Calcium signalling in glial cells. *Trends Neurosci.* **19**: 346-352.
- Wang, Z., Tymianski, M., and Nedergaard, M. 1997. Impact of cytoplasmic calcium buffering on the spatial and temporal characteristics of intercellular calcium signals in astrocytes. *J. Neurosci.* **17**: 7359-7371.

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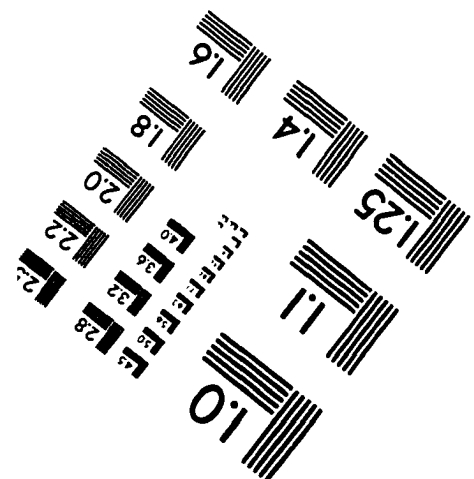
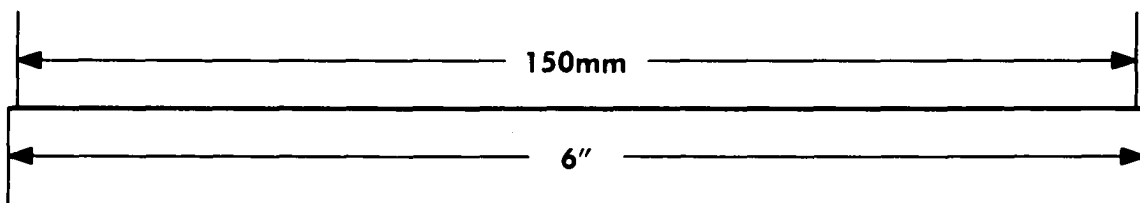
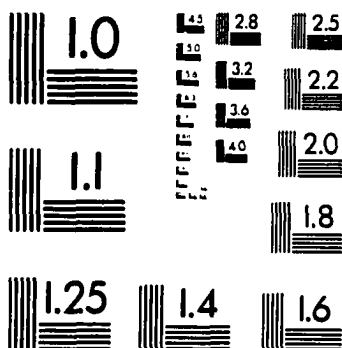
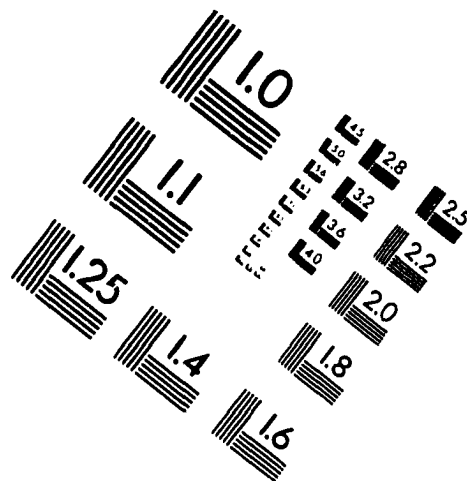
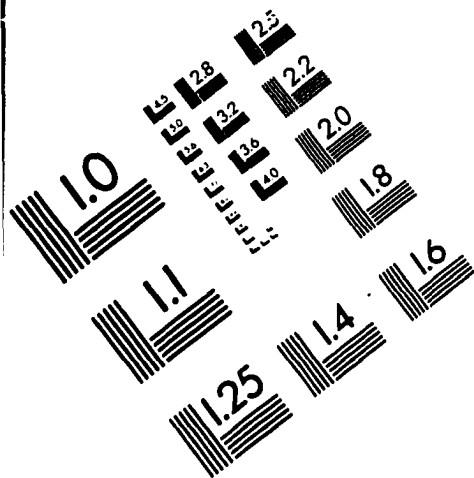
PROFESSIONAL PUBLICATIONS:

- Kaushik N.A., Sanzgiri R.P., Patil M.S. and Pandey V.N. (1992) Cloning and overproduction of the large proteolytic fragment (Klenow) of *Escherichia coli* DNA polymerase I. *Current Science* 64: 102-107
- Pandey V.N., Kaushik N.A., Sanzgiri R.P., Patil M.S., Modak M.J. and Barik S. (1993) Site directed mutagenesis of DNA polymerase I (Klenow) from *Escherichia coli*: The significance of arginine 682 in catalysis. *Euro J Biochem.* 214: 59-65
- Du C., Sanzgiri R.P., Shaiu W.-L., Choi J.-K., Hou Z., Benbow R.M. and Dobbs D.L. (1995) Modular structural elements in the replication origin region of *Tetrahymena* rDNA. *Nucleic Acids Res* 23: 1766-1774
- Araque A., Parpura V., Sanzgiri R.P. and Haydon P.G. (1998) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Euro J Neurosci* 20: 2129-2142
- Araque A., Sanzgiri R.P., Parpura V. and Haydon P.G. (1998) calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J Neurosci* (in press)
- Araque A., Sanzgiri R.P., Parpura V. and Haydon P.G. (1998) Astrocyte-induced modulation of synaptic transmission. *Canadian J Physiol Pharmacol* (in press)
- Araque A., Parpura V., Sanzgiri R.P. and Haydon P.G. (1998) Tripartite synapses: Glia the unacknowledged partner. *Trends in Neurosci* (submitted)
- Sanzgiri R.P., Araque A. and Haydon P.G. (1998) Prostaglandin-E₂ stimulates glutamate-dependent astrocyte-neuron signaling in cultured hippocampal cells. (in preparation)

SELECTED ABSTRACTS:

- Sanzgiri R.P., Trudeau L.-E. and Haydon P.G. (1996) Overexpression of synaptic proteins in primary cultured astrocytes. *Soc Neurosci Abstr* 22:583
- Sanzgiri R.P., Trudeau L.-E. and Haydon P.G. (1997) Synaptotagmin I enhances transferrin uptake in primary cultured astrocytes. *Soc Neurosci Abstr* 23:1171
- Sanzgiri R.P., Araque A. and Haydon P.G. (1998) Prostaglandin-E₂ stimulates an astrocyte-evoked NMDA and non-NMDA glutamate receptor dependent slow inward current in neurons. *Soc Neurosci Abstr* (in press)
- Araque A., Sanzgiri R.P., Parpura V. and Haydon P.G. (1998) Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of the miniature synaptic currents in cultured hippocampal neurons. *Soc Neurosci Abstr* (in press)

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